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Abstract

Hypoxia regulates physiological functions including erythropoeisis, ventillatory drive, angiogenesis, vascular tone, and glycolytic function - all which are essential for systemic and cellular adaptation to lowered oxygen tension. This is mediated in part through induction of a hypoxia-inducible transcription factor (HIF-1) which is instrumental in the regulation of genes such as vascular endothelial growth factor (VEGF) and erythropoietin (EPO). The purpose of the following work was to identify specific elements of the hypoxic signaling pathways involved in HIF-1 activation in a glial derived cell line (U87 glioma) using gel shift analysis. Since lowering of available oxygen effectively lowers the production of reactive oxygen species (ROS), this shift in ROS production could be the hypoxic signal which mediated HIF-1 induction. Exogenously added H_2O_2 prevented HIF-1 activation by hypoxia, and catalase, an enzyme which depletes H_2O_2 , was found to activate HIF-1 DNA binding activity under normoxic conditions. Reduction of mitochondrial produced H_2O_2 , using thenoyltrifluoroacetone (TTFA), induced HIF-1 DNA binding activity under normoxia. These findings suggest that a decrease in the production of

ROS such as H_2O_2 during hypoxia may serve as an upstream signal for hypoxic gene expression in glioma cells. Given the evidence which supports the role of protein kinase C (PKC) in ischemic preconditioning, an adaptive phenomena of cardiac and glial cells, the involvement of PKC in HIF-1 activation was evaluated. The involvement of PKC was supported by the following findings: **1)** chelerythrine chloride (CHEL), a selective PKC inhibitor dose dependently extinguished HIF-1 binding activity; **2)** phorbol myristate acetate (PMA) treatment induces HIF-1 binding activity which can be blocked by CHEL; and **3)** hypoxia promotes translocation of PKC α from cytosolic to membranous cell fractions. Consistent with the findings that hypoxia stimulates HIF-1 in U87 cells by lowering reactive oxygen species (ROS), we report that catalase stimulation of HIF-1 binding is also blocked by CHEL. Furthermore, hypoxia induced PKC translocation is blocked by exogenously added H_2O_2, and catalase treatment induces translocation even under normoxia. Taken together these results implicate the involvement of PKC in U87 hypoxic signaling; and place redox mediated signaling events upstream of PKC enzyme activity in HIF-1 activation.

HYPOXIA INDUCIBLE FACTOR 1 (HIF-1) ACTIVATION IN U87 GLIOMA CELLS INVOLVES A DECREASE IN REACTIVE OXYGEN SPECIES PRODUCTION AND PROTEIN KINASE C ACTIVITY

by

Robert A. Forbes

Dissertation submitted to the Faculty Committee of the Neuroscience Program of the Uniformed Services University of the Health Sciences in partial fulfillment of the requirements for the degree of Doctor of Philosophy
1998

Dedication

To my loving wife, Shawn. The light at the end of my tunnel.

Acknowledgements

Most of all I thank my family for their tremendous moral and financial support and guidance through the many years of my education.

I appreciate the guidance and support of each of my committee members: Cinda Helke, Jack McKenzie, Regina Armstrong , Ajay Verma, and Aviva Symes.

My mentor Ajay Verma has provided a rich environment in which to learn and grow as a scientist. His patience and intellectual curiosity, as well as his unique approach set a great example to all members of his lab. I have been privileged to share in at least a fraction of his progressive vision.

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List of Abbreviations

2-AP	2- aminopurine
Ab	Antibody
Allo	Allopurinol
AP-1	Activator Protein -1
ATP	Adenosine triphosphate
BIS II	Bisindolmaleimide II
CAT	Catalase
CH	Chelerythrine Chloride
CHX	Cycloheximide
DFM	Curcumin
DFX	Desferrioxamine
DNA	Deoxyribonucleic Acid
DPI	Diphenyliodinium
DPPD	Diphenylphenylenediamine
DTH	Dithionite
EMSA	Electrophoretic mobility shift assay
EPO	Erythropoietin
Fe	Ferrous Chloride
H2O2	Hydrogen peroxide
Herb A	Herbimycin A
HIF-1	Hypoxia Inducible Factor-1
HRP	Horseradish peroxidase
HVA	Homovanillic Acid
IAP	Immobilized Alkaline Phosphatase
IBU	Ibuprofen

KCN	Potassium Cyanide
MUT	Mutant
NFκB	Nuclear Factor κ β
O2	Oxygen
PAP	Potato acid Phosphatase
PMA	Phorbol myristate acetate
PKA	Protein Kinase A
PKC	Protein Kinase C
PKG	Protein Kinase G
RNA	Ribonucleic Acid
ROI	Reactive oxygen intermediate
ROS	Reactive oxygen species
TK	Tyrosine kinase
TTFA	Thenoyltrifluoroacetone
VAN	Sodium Vanadate
VEGF	Vascular endothelial growth factor
WT	Wild type

STATEMENT OF PURPOSE

Hypoxia has a profound effect on virtually all aspects of cellular function, yet the mechanism by which cells sense and respond to changes in oxygen tension has not been elucidated. The mortality and morbidity associated with cardiovascular and cerebrovascular disease, respiratory disease, and neoplastic disorders can be largely attributed the effects mediated by hypoxia. Despite the wealth of knowledge available on the mechanisms of hypoxic injury in the brain and other organs, relatively little is known about how to protect such tissues from this insult. Several lines of evidence now suggest that the protection against hypoxic injury may involve the recruitment or augmentation of autoprotective mechanisms. Indeed, glial cells have been shown to induce such autoprotective mechanisms when challenged with hypoxia. Hypoxia induced gene expression in glial cells results in the increased production of several adaptive proteins including VEGF (Goldberg and Schneider, 1994) and EPO (Goldberg and Schneider, 1994, Marti et al., 1997, Marti et al., 1996, Masuda et al., 1994) which increase brain oxygen delivery in addition to having direct neuroprotective effects (Fern et al., 1996, Morishita et al.,

1997). The identification of a hypoxia inducible transcription factor known as HIF-1 (Semenza and Wang, 1992) has focused attention on a major switch in the hypoxic signaling machinery which may mediate such hypoxic adaptations through upregulation of gene expression. The objective of the following work is to elucidate the mechanisms by which glial cells sense lowered oxygen tension and how the hypoxic signal is transduced to modulate HIF-1 activation. This work was performed in a human astrocytoma cell line (U87-MG) because these cells have been shown to increase their production of VEGF in response to hypoxia (Mukhopadhyay et al., 1998). Furthermore, HIF-1 has been shown to instrumental in the hypoxic regulation of the VEGF gene (Forsythe et al., 1996). Therefore, these cells are an appropriate model in which to examine hypoxic signaling pathways in glial derived cells.

INTRODUCTION

I. MODULATION OF GENE EXPRESSION BY HYPOXIA

A. *HYPOXIA REGULATED GENES*

Several genes have been found to be induced by hypoxia in a variety of different tissues (Helfman and Falanga, 1993). These genes can be divided into 3 general categories based on the direct or indirect actions of their encoded proteins. The first group includes genes which are favorable for the adaptation of the whole organism to general hypoxia such as EPO and tyrosine hydroxylase (TH). A second class comprises local acting factors that ensure the survival of tissues exposed to local hypoxia due to high oxygen consumption, reduced blood supply, or injury. VEGF, a potent angiogenic factor which promotes neovascularization in affected tissues (Plate et al., 1994, Plate and Risau, 1995, Shweiki et al., 1992), interleukin 1 α (Shreeniwas et al., 1992) platelet-derived growth factor beta chain (Kourembanas et al., 1990). The third group consists of intracellular factors involved in the adaptation of the cell to hypoxia such as glycolytic enzymes Aldolase A, Phosphoglycerate kinase 1, lactate dehydrogenase A, pyruvate kinase M, which provide ATP through anaerobic glycolysis (Semenza et al., 1994); or, transcription factors of the Jun and Fos family which are induced by hypoxia in cardiac myocytes and hepatoma

(Goldberg and Schneider, 1994, Webster et al., 1994). Due to the limited scope of this introduction only hypoxic induction of VEGF and EPO will be examined.

Erythropoietin

Induction of EPO gene transcription by hypoxia (Beru et al., 1986) is probably the most studied and best characterized example of hypoxia regulated gene expression. In mammals, red blood cell mass is regulated by a 30kDa glycoprotein hormone, EPO required for the survival of erythroid progenitors. Decreased oxygen carrying capacity of the blood either from anemia, acute hemorrhage, decreased ambient O_2 tension, or decreased O_2 -hemoglobin dissociation, stimulates up to a 1000-fold increase in EPO messenger RNA and protein synthesis in liver and kidney cells.

The benefits of this systemic adaptive response are obvious. Less well characterized, however, is the role and possible benefit of EPO in the brain. Marti et al. (1996) demonstrated EPO mRNA for the protein and receptor in the rodent brain, monkey brain, and in biopsies from the human temporal lobe. Hypoxia exposure enhanced EPO mRNA levels in the monkey brain, as did anemia in the mouse brain. When incubated at 1% oxygen, astrocytes isolated from murine brain showed 100-fold increase

in EPO mRNA, whereas separate isolates of microglia did not have baseline production (Marti et al., 1996, Masuda et al., 1994). Further work by this group demonstrated that human cerebral spinal fluid (CSF) EPO immunoreactivity levels did not correlate with serum levels, suggesting the impermeability of this factor to the blood brain barrier (Marti et al., 1997). Morishita and colleagues recently showed that addition of recombinant EPO to hippocampal and cerebral cortical neurons protected against glutamate induced neurotoxicity. This effect was reversible by the addition of a soluble EPO receptor to the culture medium (Morishita et al., 1996). Together these studies support the involvement of astrocyte-derived EPO in the CNS neuroprotective response to hypoxia.

Cloning of the human and murine EPO gene, the availability of a convenient EPO producing hepatoma cell line (Hep3B), and the fact that the hypoxic induction of EPO is considerably greater than other hypoxia responsive genes have facilitated the study of hypoxic regulation of gene expression. Induction of EPO in Hep3B cells depends primarily on increased transcription (Goldberg et al., 1991). However, EPO mRNA stability is also an important level of regulation (Rondon et al., 1991, Rondon et al., 1995). Several key regulatory regions of the EPO gene have been identified in the 3′ and 5′ flanking regions, namely a

hypoxia responsive element (HRE) which binds a hypoxia inducible transcription factor HIF-1 found in the immediate 3′ flanking enhancer region (Semenza et al., 1991). This 40 base pair region confers hypoxia inducible gene expression in a variety of transfected cel lines (Beck et al., 1991, Blanchard et al., 1992, Madan and Curtin, 1993, Pugh et al., 1994, Pugh et al., 1991, Semenza et al., 1991, Semenza and Wang, 1992). Further examination of this region revealed that the enhancer is functionally tripartite. HIF-1 binds a highly conserved 8-mer region in the 5′ enhancer (Beck et al., 1993). The lesser conserved middle portion of the enhancer, which is necessary for the hypoxic induction of EPO (Semenza and Wang, 1992) and other HIF-1 regulated genes (Ebert et al., 1995, Firth et al., 1995) consists of several CA repeats but protein binding to this region has not yet been demonstrated. The highly conserved 3′ region is a repeat of a consensus element thought to be a member of the steroid hormone receptor superfamily, hepatocyte nuclear factor 4 (Galson et al., 1995).

Vascular Endothelial Growth Factor

Oxygen delivery to the mammalian brain is under precise and dynamic control so that most of the brain tissue is exposed to the minimal necessary amount of O_2. Adaptation to prolonged hypoxia results in the maintenance of adequate oxygen delivery

to the brain. This is attributable, in part, to increased oxygen carrying capacity of the blood via polycythemia. Tissue oxygen availability can also be modulated by shortening the diffusional distance of O_2 from blood cells to neurons and glia through an increase in tissue capillary density. Prolonged hypoxic treatment of adult rats increases brain vascularity with significant proliferation of brain capillaries (Harik, 1992). Study of autopsy specimens from brains of South American Indians living at high altitudes and chronically exposed to hypoxia has also revealed increased microvessel density (Cervos-Navarro et al., 1987). The ability of altered O_2 levels to modulate physiologic changes in the brain is appreciated in some pathological scenarios and a more comprehensive understanding of angiogenesis in the adult central nervous system may have significant clinical impact. Infarcted human brain tissue shows statistically significant increase in the number of microvessels when compared with the contralateral area in the normal hemisphere (Krupinski et al., 1994, Krupinski et al., 1993). The active angiogenesis is more developed in the ischemic penumbral region and patients whose brains were found to have higher peri-infarct blood vessel counts displayed longer survival (Krupinski et al., 1994). Positron emission tomography and single photon emission tomography studies have also implied that a higher blood vessel

density indicates a better prognosis in stroke patients (Marchal et al., 1993). While the angiogenic factors involved in brain hypervascularization are not yet elucidated it is likely that VEGF is involved.

Recent public attention has been given to the substantiation of the long suspected necessity of angiogenic factor activity in tumor survival (Claesson-Welsh et al., 1998, O. Reilly et al., 1995, Weidner et al., 1992). The core region of gliomas where pO_2 levels are significantly lower than that in superficial layers of the mass, have been shown to produce VEGF, and endogenous angiogenesis inhibitors are proposed to reduce tumor size by inhibiting the hypoxic adaptation of these tissues. Numerous studies indicate that VEGF expression is markedly stimulated by hypoxia both in vitro (Shima et al., 1995, Shweiki et al., 1992) and in vivo (Minchenko et al., 1994a). Indeed, there are several other cellular models of clinical relevance in which hypoxic VEGF induction is proposed to promote cellular survival. VEGF induction by hypoxia in retinal cells promotes proliferation and mediation of intraocular neovascularization resulting from ischemic retinal diseases (Pe'er et al., 1995). Additionally, rat cardiac myocytes, which demonstrate development of hypoxic tolerance when challenged with sublethal hypoxic exposure (Silverman et al., 1997) have

been shown to upregulate production of both VEGF mRNA and protein in response to hypoxia (Levy et al., 1995).

Two hypoxia responsive cis-acting elements have been identified in the VEGF gene, one in the 3' flanking region of the gene which is highly homologous to the 3' EPO enhancer (HIF-1 binding site), the other in the 5' region which lacks homology with the EPO enhancer element (Liu et al., 1995, Minchenko et al., 1994b). The HRE of the 3′ enhancer confers hypoxia inducible reporter gene expression in transfected C6 glioma cells (Damert et al., 1997). However, the enhancer fragment containing the HIF-1 binding site is not sufficient to confer full hypoxia responsivity. Whereas, activator protein (AP-1) binding upstream of the HRE is not capable of conferring hypoxia responsiveness on its own, fragments containing AP-1 and HIF-1 binding sites resulted in potentiated reporter gene expression under hypoxia (Damert et al., 1997).

Mechanisms of Hypoxic Gene Expression

Independent pharmacological investigation of the induction of these and other hypoxia sensitive genes has revealed multiple mechanistic similarities. Important clues about the oxygen sensing mechanisms involved in the hypoxic induction of EPO and VEGF gene expression were provided by Goldberg and

colleagues (Goldberg et al., 1988, Goldberg and Schneider, 1994). Hypoxic stimulation of EPO protein in hepatoma cells and VEGF protein in C6 glioma cells was shown to be RNA and protein synthesis dependent. Cyanide was unable to mimic the hypoxic induction, suggesting that cells were not responding to the consequences of reduced oxidative phosphorylation or a change in metabolic status. EPO and VEGF mRNA production was markedly enhanced by exposure to salts of the divalent metals cobalt, nickel and manganese, while hypoxic gene stimulation was blocked by the coexposure of cultures to 10% CO. Hypothetically, these metals substitute for ferrous iron at the core of a putative heme based oxygen sensor resulting in oxy to deoxy conformational shift of the protein (Figure 1B,C)

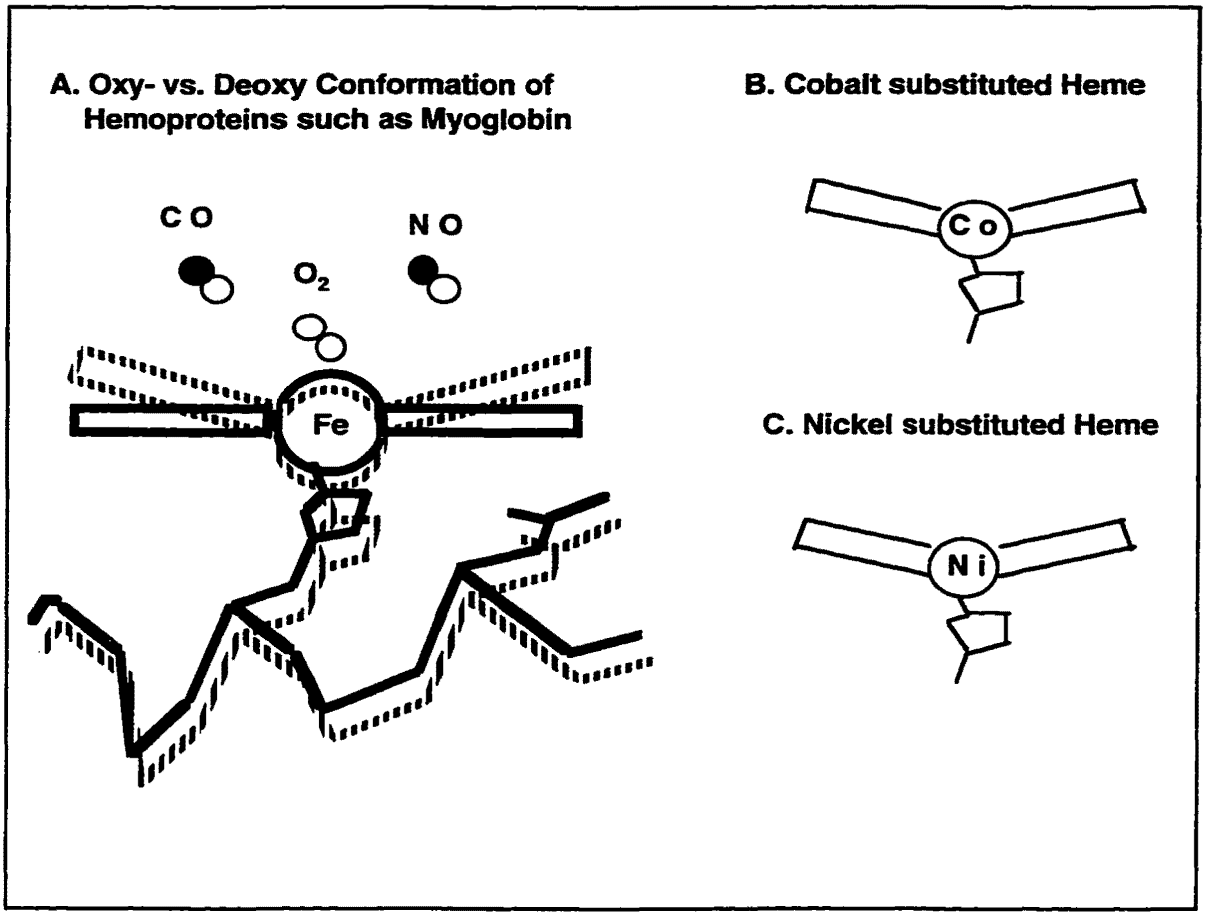


Figure 1: Proposed Heme -Based Oxygen Sensor. Oxygen binds iron at the core of the proposed hemoprotein, holding the protein in the oxy conformation (dashed lines). High affinity binding of CO or NO to iron blocks the oxy-deoxy shift. Substituted cobalt (Co) or nickel (Ni) for iron at the core of the hemoprotein do not bind oxygen, CO, or NO and thus do not acquire an oxy conformation.

This idea is further supported by the finding that agents which interfere with heme synthesis, DFX and 4,6-dioxoheptanoic acid, transiently induce EPO production. Perhaps the strongest evidence in favor of a heme protein oxygen sensor comes from experiments which utilize CO. CO is a chemically inert gas which binds with high specificity to ferrous heme groups in hemoglobin and myoglobin (Figure 1A) , as well as certain cytochromes and heme proteins. Coincubation of Hep3B cells with 10% CO dramatically reduced the hypoxic induction of Epo protein, but not the induction by cobalt and nickel (Goldberg et al., 1988). Also, hypoxia induced expression of VEGF and EPO mRNA (Goldberg and Schneider, 1994) and phosphoenoylpyruvate carboxykinase (Kietzmann et al., 1993) is reduced by CO co-treatment. Although the evidence presented is in favor of a heme based oxygen sensor, the identity and functionality of such a protein or complex of proteins remains unsubstantiated.

Although both EPO and VEGF gene expression are regulated to some extent at the level of mRNA stability, hypoxia mediated transactivation of both genes involves HIF-1 activity, and both demonstrate similar mechanistic pathways of induction. These findings provide support for the hypothesis that the mechanism(s) by which hypoxia is sensed and transduced at a

molecular level may be highly conserved and tightly regulated. A clear understanding of these mechanisms may offer insight into the development of novel therapeutic strategies to rescue ischemic tissues and/or to halt angiogenic dependent tumor growth.

B. HYPOXIA INDUCIBLE TRANSCRIPTION FACTOR (HIF-1):

The single most important advancement in the quest to delineate mechanisms involved in hypoxia regulated gene expression was the discovery and subsequent characterization and purification of HIF-1 (Semenza et al., 1991, Semenza and Wang, 1992, Wang et al., 1995a, Wang and Semenza, 1993a, Wang and Semenza, 1993b, Wang and Semenza, 1995). Since the identification of HIF-1, many investigators have sought to link its activity to general hypoxic gene expression, for reasons important to basic and clinical sciences. There has been much progress on this front, as demonstrated by identification of homologous HIF-1 binding sites in various genes (Table 1); and, as demonstrated by hypoxia inducible reporter gene expression conferred in tranfectants with HRE containing constructs. HIF-1 is now recognized as a central regulator in the mediation of hypoxic adaptation at the systemic and cellular levels.

Table 1

Hypoxia-inducible gene	HIF-1 binding site	Reference
Erythropoietin	TAC**GTG**CTGT	Wang et al, 1993
Phosphofructokinase L	TAC**GTG**CTGC	Semenza et al, 1994
Aldolase A	GAC**GTG**ACTC	Semenza et al, 1996
Phosphoglycerate Kinase 1	GAC**GTG**CGGC	Semenza et al, 1994
Enolase 1	CAC**GTG**CGCC	Semenza et al, 1994
Lactate Dehydrogenase A	CAC**GTG**ACGG	Firth et al, 1995
Glucose Transporter 1	GGC**GTG**CCGT	Ebert et al, 1995
Vascular Endothelial Growth Factor	TAC**GTG**GGCT	Liu et al, 1995
Inducible Nitric Oxide Synthase	TAC**GTG**CTGC	Melillo et al, 1995
Retrotransposon VL30	TAC**GTG**CT	Estes et al, 1995
Heme Oxygenase 1	GAC**GTG**CTGG	Lee et al, 1997
Transferrin	TAC**GTG**CGCT	Rolfs et al, 1997

HIF-1 binds the sequence 5′-TACGTGCT-3′ in the 3′ flanking region of the Epo enhancer, and this site-specific binding activity facilitated its purification (by affinity purification) and subsequent cloning of its genes (Wang and Semenza, 1995). It is an a/ß heterodimer, with each subunit containing a basic-helix-loop-helix (bHLH) motif and a PAS protein-protein interaction domain(derived from Per, Aryl hydrocarbon receptor, and Sim, the first three transcription factors found with this domain) found in a rapidly expanding family of known, or suspected transcription factors including the mammalian proteins aryl hydrocarbon receptor (AHR) and aryl hydrocarbon receptor nuclear translocator (ARNT). The HIF-1ß subunit is identical to ARNT, which can also serve to form a heterodimer with AHR in the cellular response to environmental toxins such as dioxin. Since HIF-1ß(ARNT) is involved in the induction of at least two distinct pathways, HIF-1 α is the critical hypoxia-specific component of the heterodimer transcription factor. In the past year HIF-1 has been shown to require heterodimerization with ARNT to acquire DNA binding and transactivational activity (Huang et al., 1996). Arany and colleagues have also recently demonstrated the critical importance of the transcriptional co-activators p300/CBP (CREB binding protein) in HIF-1 functions (Arany et al., 1996). p300/CBP are homologous transcriptional adapter

proteins, active in multiple transcriptional events, and are thought to act, at least in part, as signaling links between specific DNA-bound transcription factors and the basal transcriptional machinery. ARNT and HIF-1 α mRNA have been shown to be constitutively expressed in many cell lines and tissues under normoxia (Huang et al., 1996). Both proteins are continuously produced but while ARNT protein can be detected under normoxia, HIF-1 α levels are negligible as it is rapidly proteolyzed by the ATP dependent ubiquitin- This degradation is somehow disrupted during hypoxia allowing HIF-1 protein to accumulate, dimerize with ARNT to form HIF-1, translocate to the nucleus, interact with p300/CBP, and activate the transcriptional machinery of hypoxia inducible genes (Arany et al., 1996). Deletion analysis has demonstrated two distinct regions in the C-terminal portion of HIF-1 to be key for proteosome and calpain protease pathways regulation by hypoxia (Salceda and Caro, 1997). (Figure 2)

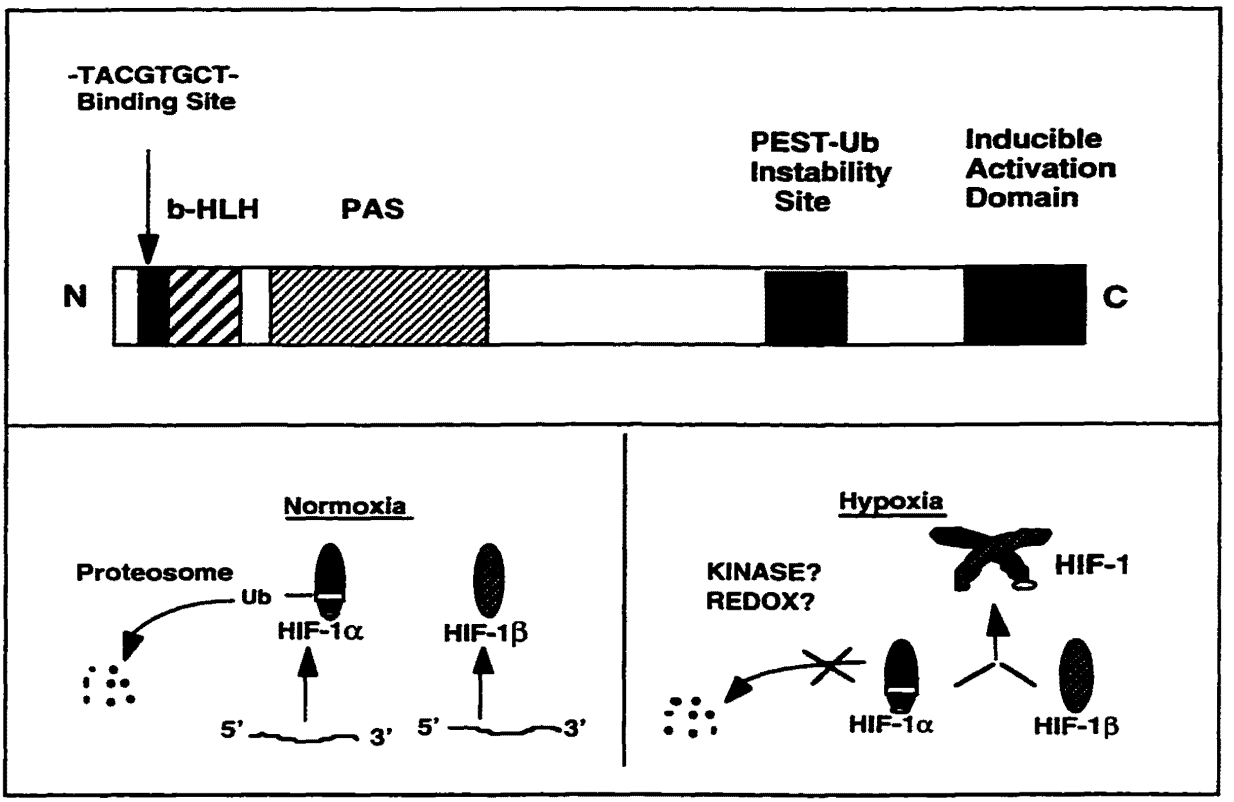


Figure 2: Sites of regulation of HIF-1 α (TOP) and proposed mechanism for HIF-1 α regulation through protein stability (BOTTOM).

One of these has PEST sequences found in many short lived proteins and confers sensitivity to rapid degradation while regulatory functions of the other site are unknown (Figure 2). The general importance of HIF-1 as a transcriptional activator was underscored by the finding that it can be induced by hypoxia in several non-EPO producing mammalian cells including human 293 embryonic kidney and HeLa cervical

carcinoma cells, mouse Ltk- fibroblasts, and C2C12 skeletal myoblasts, Rat1 fibroblasts, and Chinese hamster ovary (CHO) cells. The Epo enhancer region containing the HIF-1 binding site can also mediate hypoxia-inducible reporter gene expression in non-Epo-producing cells (Semenza et al., 1994). Widespread importance of HIF-1 in hypoxic adaptation is now appreciated (Guillemin and Krasnow, 1997) and is underscored by the nonviability of HIF-1 -/- mutant mice (Iyer et al., 1998), yet the sensing of O_2 and the signal transduction pathways involved in HIF-1 activation remain poorly understood.

II. MECHANISMS OF HIF-1 ACTIVATION

Given the ubiquitous nature of HIF-1 in modulating the cellular response to hypoxia, the past eight years of research has produced a steadily increasing number of publications devoted to delineating the pathways critical to its regulation. At present HIF-1 activation appears to occur through modulation of cellular redox status, either by direct modification of HIF-1 α protein (Huang et al., 1996), and/or through downstream events secondary to a change in cellular levels of ROS (Fandrey et al., 1997, Fandrey et al., 1994). Secondly, HIF-1 DNA binding activity requires protein phosphorylation (Wang et al., 1995c, Wang and Semenza,

1993a), though the identity of the kinase(s) involved has not been established.

HYPOXIA AND METABOLIC STATUS

The classical view of cellular adaptation to hypoxia focuses primarily on the switch from aerobic to anaerobic metabolism - the switch from oxidative phosphorylation to glycolysis. Enzymes of glycolysis are negatively regulated by metabolites of energy production (i.e. ATP), whereas they are positively regulated by products of energy expenditure (i.e. AMP). Such feed back loops endow the organism with the ability "shut down" non-essential energy expending cell functions (Hochachka et al., 1996) and turn on mechanisms which facilitate the efficient usage of available energy supplies. As mentioned earlier, several genes associated with glycolytic function are known to be upregulated by hypoxia (Wenger and Gassmann, 1997). EPO and VEGF gene expression is not induced by inhibitors if oxidative phosphorylation cyanide or azide (Goldberg et al., 1988, Goldberg and Schneider, 1994). Nor is HIF-1 activated by cyanide induced ATP depletion in hepatoma cell cultures (Semenza, 1994). The glucose transporter-1 gene however is upregulated by hypoxia and mitochondrial inhibitors rotenone and azide through distinct cis elements encoding binding sites for HIF-1 and a

serum response element, respectively (Ebert et al., 1995). Hence, hypoxia mediated regulation of certain genes may involve an integration of signaling pathways. Since cells are able to sense changes in oxygen tensions well before cellular ATP pools are depleted, the activation of hypoxia sensitive genes by distinct mechanisms may represent a graded adaptive response - the full responsivity of gene output occuring at the onset of more severe hypoxia. Although HIF-1 is not activated by exposure to cyanide, other site specific inhibitors of the mitochondrial electron transport chain that lower ATP production and lower mitochondrial ROS generation may activate HIF-1.

REACTIVE OXYGEN SPECIES IN HYPOXIC SIGNALING

All cells produce constitutive levels of ROS, and in some scenarios these are produced in sufficient quantities to cause oxidant stress or oxidative damage to the cell such as in acute chronic inflammatory diseases (e.g. rheumatoid arthritis) (Meier et al., 1990). ROS are generated as side products of electron transfer reactions involving metals such as Fe2+ or Cu2+. In addition, ROS are contributed from over 100 enzymatic sources which utilize molecular oxygen as a substrate (Halliwell, 1996) The levels of ROS are effectively buffered by several specialized enzymes including catalases,

glutathione peroxidase, and superoxide dismutase (SOD). (See Figure 3)

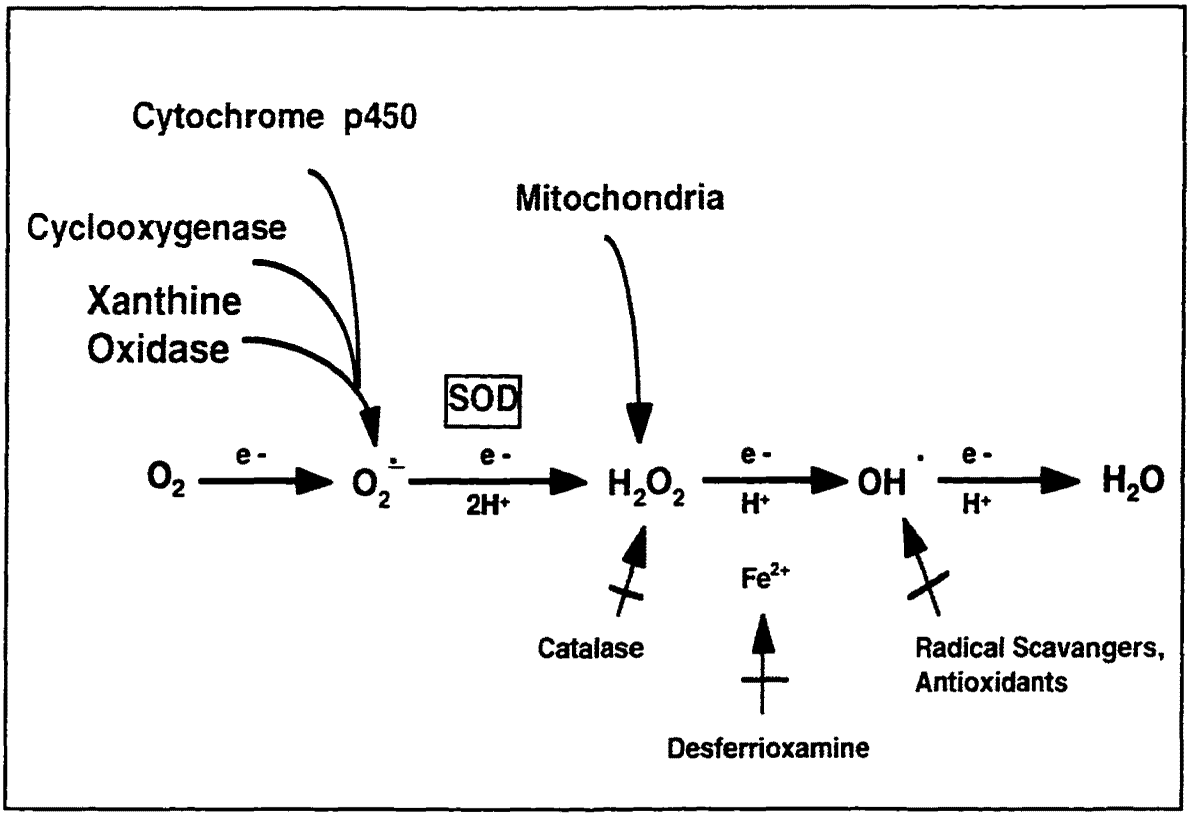


Figure 3: Production of ROS through various enzymatic sources and metal catalyzed reactions (Halliwell, 1983). (O_2^- =Superoxide; H2O2 = hydrogen peroxide; OH · = hydroxyl radical)

While scientists have traditionally viewed ROS production as waste reactions, and studied their role in cytotoxicity or cellular defense, it is becoming increasingly clear that

levels of ROS production and elimination are tightly regulated and satisfy several criteria for consideration as intracellular messenger molecules. Support for the role of ROS as second messenger molecules comes from the observations that, 1) they are synthesized when cells are stimulated by the binding of a ligand (O_2) to a "receptor", and 2) they directly modulate downstream effector molecules as in the case of sulfhydryl oxidation of transcription factor subunits [Nuclear Factor κβ (NFκB), OxyR, AP-1]. Additionally, the physical properties of ROS make them highly suitable as intra or extracellular messengers. The extreme reactive nature and short diffusion distance of hydroxyl radical makes it less well suited to transmit specific signals. Likewise, superoxide, while less reactive is a charged molecule and is unlikely to penetrate lipid bilayers. Hydrogen peroxide, on the other hand, is a relatively stable intermediate; it is lipophilic and able to traverse the plasma membrane (or mitochondrial membrane); and its enzymatic production as well as its enzymatic degradation is precisely and rapidly regulated outside the margin of toxicity.

It is well established that cellular redox changes are instrumental in the activation of several transcription factors. Changes in cellular redox status is a critical

determinant in the formation of the AP-1 complex (c-Fos/c-Jun complexes or c-Jun homodimers). AP-1 is activated by antioxidants (Muller et al., 1997) and by thioredoxin (Hirota et al., 1997) in various cell types, whereas exogenous H2O2 and sulfhydryl oxidants block activation (Meyer et al., 1993). Replacement of cysteines in the binding domains of Fos and Jun with serines confers enhanced activity which is resistant to oxidant inhibition (Hirota et al., 1997). Recent reports show redox factor-1 induction by thioredoxin modulates AP-1 activity, rather than direct ROS modulation of AP-1 (Hirota et al., 1997). NFκB is a multisubunit transcription factor involved in the activation genes associated with inflammatory response (Baeuerle, 1991). NFkB activation, associated with release of the inhibitory subunit IkB, is stimulated by various agents including cytokines, lipopolysaccharide, phorbol esters, and calcium ionophores which are known to increase cellular levels of ROS (Meyer et al., 1993, Schreck and Baeuerle, 1994, Schreck et al., 1991). NFκB activation is stimulated by exogenously added H_2O_2 and activation by various agents is inhibited by cotreatment with antioxidants N-acetyl-L-cysteine (NAC) (Schreck et al., 1991). Increase ROS production is therefore thought to directly or indirectly modulate IkB release in the

cytosolic compartment and consequent activation of NFkB binding activity.

Given the relationship between cellular O_2 availability and ROS levels, redox processes have also been implicated as a candidate in O_2 sensing and hypoxic signaling (Bunn and Poyton, 1996). Normoxic production of superoxide and H_2O_2 has thus been proposed as an inhibitory signal for hypoxic gene expression by promoting HIF-1 degradation (Huang et al., 1996) or a direct modulation of HIF-1 α , altering HIF-1 DNA binding properties (Wang et al., 1995b). A decrease in reactive oxygen intermediate (ROI) production is thus envisioned as the initial hypoxic signal and is proposed to activate HIF-1 binding activity. Fandrey and coworkers (1994) showed that hypoxic induction of Epo in Hep3B cells could be inhibited the addition of H_2O_2, menadione (an agent which increases intracellular H_2O_2 levels) or aminotriazole, an inhibitor of catalase. Catalase and N-(2-mercaptopropionyl)-glycine (NMPG), a reducing antioxidant that decreases H_2O_2 concentration, have also been shown to increase tyrosine hydroxylase mRNA levels (Kroll and Czyzyk-Krzeska, 1998). These results suggest that there is indeed an inverse relationship between H_2O_2 levels and hypoxic gene expression.

Although the source of continuous ROI production in normoxic cells remains undetermined, an NADPH oxidase, similar to that found in neutrophils, has been proposed by many investigators to serve as the oxygen sensor. This is mainly based on the demonstration of immunoreactivity for some components of this enzyme complex in some (but not all) cells which demonstrate hypoxic gene expression (Gorlach et al., 1993, Kummer and Acker, 1995). DPI potently inhibits oxygen free radical production by NADPH oxidases and other flavin-containing hemoproteins. Unlike other oxygen radical depletors however, this compound potently inhibits (IC_{50} <1υM) hypoxic gene expression rather than stimulating it (Gleadle et al., 1995). This enigma has prevented a clear conceptualization of the oxygen sensor and may be explained by some nonspecific actions of DPI since this drug also modifies cation channels, nitric oxide synthase, and mitochondrial flavoproteins (Gatley and Sherratt, 1976).

How might H_2O_2 exert inhibitory effects on the hypoxia signaling pathway? Several lines of investigation support a role for reactive oxygen metabolites and redox mechanisms in signal transduction mechanisms including modulation of protein ubiquitinylation (Jahngen-Hodge et al., 1997, Shang

et al., 1997) and protein phosphorylation via regulation of a protein kinase and protein phosphatase activity (Whisler et al., 1995). HIF-1 activity may be modulated by increasing the stability of the HIF-1 α protein through direct redox modification (Huang et al., 1996, Salceda and Caro, 1997, Wang et al., 1995b), as has been demonstrated with other eukaryotic transcription factors (Muller et al., 1997, Schreck et al., 1991) and bacterial oxygen responsive transcription factors such as OxyR (Kullik et al., 1995, Zheng and Storz, 1998).

Additionally, ROS may play a role in modulation of potassium (K+) channels known to exist in specialized excitable cells of the carotid body, bronchial neuroepithelial bodies, and pulmonary vascular smooth muscle cells (Buckler, 1997, Wang et al., 1996, Wang et al., 1997, Youngson et al., 1994). This concept has been investigated most vigorously in pulmonary vascular smooth muscle (PVSM) which constricts under hypoxia to shunt blood from hypoxic alveolar regions to better oxygenated alveolar regions (Archer et al., 1993). Lowered oxygen tension triggers an inhibition of K+ channel conductance (probably through a decrease in open probability of the channel), activation of voltage dependent calcium channels and subsequent muscle constriction (Harder et al.,

1985). It is now appreciated that more than one type of K+ channel may be involved, including TEA (Post et al., 1996, Stys et al., 1998), 4-aminopyridine (Clapp and Gurney, 1991) and charybdotoxin (Yuan et al., 1995) sensitive channels. PVSM K+ channel conductance may also be modulated by cellular redox changes as evidenced by inhibition of the channel with reduced glutathione (GSH) and NADH (Archer et al., 1993, Post et al., 1996, Yuan et al., 1995). DPI an inhibitor of NADPH oxidase inhibits vascular smooth muscle K+ and Ca2+ channel activity (Thomas et al., 1991) but also inhibits hypoxia induced pulmonary vasoconstriction (Weir et al., 1994). It is unclear as to whether these K+ channels are modulated directly by changes in O_2 tension or by secondary effects of cellular oxygen fluctuations.

ACTIVATION OF HIF-1 REQUIRES PROTEIN PHOSPHORYLATION

Other clues about the potential mechanisms in HIF-1 activation stem from work in the bacterium Rhizobium meliloti which respond and adapt to the hypoxic environment via a two-component regulatory system -involving the sensing of hypoxia by a heme-based protein with kinase activity and subsequent phosphorylation of the transcription factor FixJ which

modulates the expression of nitrogen fixation genes (Monson et al., 1995). The mammalian response to hypoxia, though more complex, also appears to require protein kinase activity, though the identity of kinase(s), target phosphoprotein(s), and sequence of transduction events involved in mammalian hypoxic signaling are not well established.

Studies by Semenza and colleagues (1992) showed that dephosphorylation of nuclear extracts from hypoxic hepatoma cells extinguished the ability of HIF-1 to bind DNA. Moreover, treatment of hypoxic cultures with 2-aminopurine, a general serine threonine kinase inhibitor blocked the hypoxic induction of HIF-1. This group later reported that genistein, a general tyrosine kinase inhibitor, blocked HIF-1 induction and that an inhibitor of tyrosine phosphatases (sodium vanadate) increased the basal levels of HIF-1 activity (Wang et al., 1995c). Others showed that genistein and PD098059 a selective MEK (MAP kinase kinase) inhibitor, block cobalt, DFX, and hypoxia mediated reporter gene expression in transfected Hep3B cells, but PD098059 failed to block HIF-1 DNA binding activity (Salceda et al., 1997). C-Src, a tyrosine kinase, has been implicated in HIF-1 induction since it is activated by hypoxia, Furthermore, hypoxic induction of VEGF gene expression is markedly reduced in c-src kinase

deficient cells (Mukhopadhyay et al., 1995). However, recent reports suggest that src kinase is not involved in HIF-1 activation since it requires near anoxic conditions for activation, whereas HIF-1 is robustly induced by 1% O_2. Src wild type and src deficient cells both demonstrate HIF-1 activation by hypoxia with a similar time course of induction. Furthermore, src kinase expression is not required for hypoxia mediated reporter gene expression, or for transcriptional activation of VEGF, Enolase 1 (Jiang et al., 1997), EPO, or Glucose Transporter-1 (Gleadle and Ratcliffe, 1997).

The ability of mild hypoxia to tolerize tissues to further hypoxic episodes is known as hypoxic tolerance. The clinical relevance of hypoxic tolerance is perhaps best appreciated in cardiac preconditioning. Brief bouts of ischemia or hypoxia markedly protect heart cells from subsequent major ischemic episodes (Murry et al., 1986). While the mechanisms for adaptation in these models remain unknown, the activation of PKC has been shown to play a crucial role. Indeed, a major role for PKC has been implicated in eliciting the autoprotective adaptations associated with hypoxic/ischemic preconditioning in CNS white matter as well as in myocardium (Cleveland et al., 1996, Fern et al., 1996) Several hypoxia tolerant animals have also been reported to utilize PKC-

dependent biochemical adaptations for survival, although the exact nature of these is unknown (Storey, 1996). The role of PKC in cell signalling is complicated by the several different isoforms (at least 11) playing tissue specific roles in different cellular subcompartments and by PKC activating processes leading to subsequent PKC downregulation. The PKC isoform (s) involved in glioma hypoxic signaling probably belongs to the conventional group consisting of PKC α , β and γ, since these are the major forms found in glia (Misra-Press et al., 1992). Involvement of HIF-1 in either cardiac or glial models of tolerance has not been examined. Isolated cardiac myocytes are known to increase VEGF gene expression in response to hypoxia, both *in vitro* and *in vivo* (Levy et al., 1995, Minchenko et al., 1994a), which is mimicked by cobalt and nickel (Ladoux and Frelin, 1994, Minchenko et al., 1994a). Recently, phorbol myristate acetate (PMA) was shown to stimulate HIF-1 α mRNA in primary rat cardiac myocytes (Ladoux and Frelin, 1997) further implicating PKC in cardiac adaptation to hypoxia. The role of PKC in hypoxic signaling is further supported by the finding that hypoxia induced tyrosine hydroxylase gene expression is blocked by chelerythrine chloride, a selective PKC inhibitor, in PC12 cells (Raymond and Millhorn, 1997).

This induction was also blocked by the incubation of hypoxic cultures in calcium free medium, or by treatment with intracellular chelators of calcium (BAPTA/AM) (Raymond and Millhorn, 1997).

The presented studies support the involvement of multiple kinases in hypoxic signaling. Additionally, there appears to be a dissociation between HIF-1 DNA binding and HIF-1 mediated transactivation.

SPECIFIC AIMS

Specific Aim 1:

To determine whether activation of HIF-1 in U87-MG cells occurs through a net decrease in production of ROS.

Rationale:

To further explore the concept of a specific O_2 sensing pathway, I explored the following hypotheses: 1) Since hypoxia is the removal of a stimulus which is normally available (O2), O2 or an oxygen metabolite acts in an inhibitory manner on HIF-1 activation. 2) Since lowering of available O_2 leads to a decrease in ROS production, the activation of HIF-1 is mediated by a decrease in ROS formation.

Plan:

To investigate these ideas I have chosen to alter the production of reactive oxygen intermediates using antioxidants, free radical scavengers, catalase and specific inhibitors of various cellular sources of ROS to assess the ability of these manipulations to induce HIF-1 DNA binding activity.

Specific Aim 2:

To determine if protein kinase C is involved in the hypoxic signaling pathway leading to HIF-1 activation in U87-MG cells.

Rationale:

Protein phosphorylation has been implicated in HIF-1 induction using hepatoma cells. Treatment of these hypoxia exposed cells with 2-aminopurine has been shown to abolish HIF-1 binding activity. *In vitro* dephosphorylation of "HIF-1 positive" nuclear extract extinguishes DNA binding activity. Protein kinase C activity has been shown to be important for the development of glial and cardiac tolerance to hypoxia where the involvement of HIF-1 has not been examined.

Plan:

Specific PKC inhibitors will be assayed for their ability to block HIF-1 activation in glial-derived cells. Membrane translocation of PKC α will also be examined with various treatments shown to induce and inhibit HIF-1 DNA binding activity.

MATERIALS

Reagents, Enzymes and Enzyme inhibitors:

All reagents used in general laboratory procedures were attained from Sigma Chemical Company (St. Louis, MO). All Enzymes were purchased from Boehringer Manheim (Indianapolis, IN), unless otherwise noted. Enzyme inhibitors were purchased from CalBiochem (La Jolla, CA).

Equipment:

Electrophoresis equipment: BioRad (Hercules, CA) and Novex, Inc. (San Diego, CA); Spectrophotometer: Hewlett Packard model 8453 (Rockville, MD); Fluorimeter/Luminometer: Perkin Elmer LS 50B (Norwalk, CT); Oxygen regulated Incubator: Forma Scientific (Marietta, OH); Hypoxia chamber: Billups-Rothenberg, Inc. (Del Mar, CA)

METHODS

Cell Culture

U87 MG human astrocytoma cells (ATCC# 14-HTB) and human hepatoma (HEP3B) cells were maintained in Dulbecco's minimal

essential medium (DMEM) and Minimal Essential Medium (MEM), respectively, with 3.7 g/L $NaHCO_3$, 4.5 g/L glucose, 2mM L-glutamine, 1mM nonessential amino acids, and 1mM sodium pyruvate supplemented with 50 U/ml penicillin, 50 mg/ml streptomycin, and 10% (vol/vol) heat-inactivated fetal calf serum in a humidified incubator with 5%CO_2, 20% O_2, balance N_2. Media was replaced 2-3 times per week. Subculture was accomplished by trypsinization. Expired media was removed prior to adding trypsin (0.25%) - EDTA (.03%) solution. Trypsin was removed and cultures were incubated at 37°C for approximately 2 minutes until cells detached. Fresh medium was added, and aliquots of cell suspension were transfered into new flasks at a subculture ratio was 1:3.

Hypoxia exposure and preparation of nuclear extracts from cultured cells

Cells at a level of approximately 60% confluency were subjected to hypoxia in a modular incubator chamber (Billups-Rothenberg) flushed with 1%O_2/ 5% CO_2/ 94% N_2 for 4 hours at 37°C. Cells were rinsed twice in ice-cold PBS, scraped into 5 ml PBS, and pelleted by centrifugation at 1,000 x g for 5 minutes at 4°C. Nuclear extracts were prepared by modification of a previously described procedure (Semenza and Wang, 1992). Lysis and extraction buffer contained 0.5mM

dithiothreitol (DTT), 0.4mM phenylmethylsulfonyl fluoride (PMSF), 2 υg of leupeptin per mL, 2 υg pepstatin per mL, 2 υg aprotinin per mL, and 1mM sodium vanadate. Briefly, the cell pellet was resuspended in 4 packed cell volumes (PCV) of lysis buffer (10 mM HEPES [pH 7.9] , 1.5 mM $MgCl_2$, 10 mM KCl, 1 mM EDTA, 0.1% Nonidet P-40) and allowed to incubate on ice for 8 min. The nuclei were pelleted by centrifugation at 10,000 x g for 2 min at 4°C. The nuclear pellet was resuspended in 1 PCV of extraction buffer (0.42 M NaCl, 20 mM HEPES [pH 7.9] , 1.5 mM $MgCl_2$, 20% glycerol). Nuclear debris was pelleted by centrifugation at 14,000 x g for 10 minutes at 4°C. Supernatant was frozen in a dry ice/isopentane bath and stored at -70°C until use. Protein concentration was determined using a standard Coomassie blue die based assay (BioRad).

Electrophoretic mobility shift assay

Binding reactions for DNA band shift assays were carried out in a total volume of 20 υL. 10 υg of nuclear extract was diluted in binding buffer (10 mM Tris-HCl (pH 7.5), 50 mM KCl, 50 mM NaCl, 1 mM $MgCl_2$, 1 mM EDTA, 5 mM DTT, 1 mM sodium vanadate, 0.5 mM PMSF and 5% glycerol) containing 0.2 υg

denatured calf thymus DNA. Samples were incubated at room temperature for 10 minutes. After addition of 0.2 ng radiolabeled probe, samples were placed on ice for an additional 15 min. For supershift analysis, 2 υl of HIF-1 α monoclonal antibody (OZ15 and OZ12) was added to the binding reaction and incubated for an additional 15 minutes on ice. Samples were loaded onto a 5% nondenaturing polyacrylamide gel and electrophoresed at a rate of 12.5V/cm in 0.3 X Tris-Borate-EDTA (TBE) buffer, at 4°C. Gels were dried and exposed to film for autoradiography. Films were photographed on a light box using a digital camera (Kodak, Inc.; Eastman, NY), and tagged image files were transfered to an image processing program (Adobe Photoshop). Number of replicates for each experimental treatment is reported in the figure legend for the corresponding EMSA.

Oligonucleotide probe synthesis and radiolabeling

The HIF-1 recognition element (HRE) probe (sense strand: 5'-GCCCTACGTGCTGTCTCA-3') corresponding to nt 1-18 of the 50 nt 3′ enhancer region of the published Epo gene sequence (Semenza and Wang, 1992) and mutant probes (sense strand: 5'-GCCCT**CAA**TGCTGTCTCA-3') were synthesized by Mike Flora (Uniformed Services University of the Health Sciences) using a Milligen 7500 sequencing apparatus. HRE sense and antisense

oligonucleotides were separately labeled at their 5′ end. Labeling reactions (20υL) contained 5X kinase buffer (250 mM Tris (pH 7.5), 50 mM MgCl2), 10 pmol oligonucleotide, 1 Unit 3 prime exonuclease - free T4 polynucleotide kinase (Boehringer Manheim; Indianapolis, IN), and 30 υCi γ-^{32}P ATP (3000Ci/mmol; 10 mCi/ml). The enzyme reaction was incubated for 15 min at 37°C, and then for 3 min at 95°C. To anneal sense and antisense strands, both were combined and incubated at 72°C for 10 min and allowed to gradually cool in a 37°C water bath for 1.5 hours. Separation of unincorporated label from labeled double-stranded probe was accomplished using G-50 push column chromatography (NucTrap™ , Stratagene; La Jolla, CA). Column was wetted with 70uL Sodium-Tris-EDTA (STE) buffer (20 mM Tris-HCl, pH 7.5, 100 mM NaCl, 10 mM EDTA). Radiolabeled probe was added to the column and gradually pushed through the column using an attached 10 mL luer-loc™ syringe. Column was washed once with 70 υL STE buffer. Eluate was collected in a fresh eppendorf tube. Radiolabeled probe was stored at -20C until use.

HIF-1 α & β antibodies

HIF-1 α monoclonal antibodies (OZ15 and OZ12) were a generous gift from the lab of D.M. Livingston, Boston MA. HIF-1 β antibody was purchased through Affinity Bioreagents, Inc.

Western blot for HIF-1α

Samples (20 or 50ug) of nuclear extract (prepared as described abouve) were resolved in a 4-20% SDS-gradient gel (Novex; La Jolla, CA) and transblotted on to nitrocellulose paper. After 1 h incubation with a 1% BSA/99% TBS blocking solution at room temperature, the nitrocellulose was probed with a 1:100 dilution of primary HIF-1 α monoclonal antibody (source listed above)1 hr at room temperature. To detect bound primary antibody, blots were incubated for 1 hr at room temperature with a 1:5000 dilution of an alkaline phosphatase-conjugated anti-mouse secondary antibody (Sigma, St. Louis, MO). The nitrocellulose was washed for 15 min x 3, then stained with a solution containing NBT/BCIP for 10-15 sec, washed thoroughly with H_2O and let dry. Visible bands at a molecular weight of approximately 97 kD were densitized using NIH image software, and intensities of bands from treated samples were reported as magnitude of induction as compared to the matching control sample.

Protein extraction for PKC subcellular distribution

The subcellular distribution of PKC isoforms was examined using the a widely used procedure described by Steinberg and colleagues, 1994 (Acs et al., 1997) Particulate fractions attained from cardiac myocytes using this procedure contained virtually all of the membrane-associated PKC isoforms (Goldberg et al., 1997). U87 cells plated in 75 mm culture flasks were washed 3 times with phosphate buffered saline (PBS) and then immediately lysed in 0.1 ml ice-cold homogenizing buffer (containing 20 mM Tris-HCl, pH 7.5, 2 mM EDTA, 50 υg/ml aprotinin, 48 υg/ml leupeptin, 5 υM pepstatin A, 1 mM phenylmethylsulfonylfluoride (PMSF), 6 mM β-mercaptoethanol,0.1 sodium vanadate, and 50 mM NaF), sonicated 15 sec x 2, then centrifuged at 100,000 g for 90 min. The supernatant was removed to be used as cytosolic fractions, and the pellet was resuspended in homogenizing buffer containing 1% Triton X-100 to solubilize particulate proteins. After 30 min shaking at 0°C Triton X-100-insoluble proteins were removed by centrifugation at 10,000 g for 10 min and the supernatant saved as particulate fractions. Protein concentration assay (BioRad/Bradford) was performed to assess the same total protein load in all experimental

conditions.

Western blot procedures

SDS -PAGE :

Samples from either cytosolic fraction particulate fraction were loaded with 1:1 SDS sample buffer (50 mM Tris-HCl, pH 6.8, 2% glycerol, 10% SDS, 6 mM β-mercaptoethanol, 0.05% bromophenol blue), and electrophoresed through a 15 % SDS-polyacrylamide gel at 120 mV.

Transfer:

Gels were tranferred overnight to nitrocellulose paper at 15 mA at room temperature. Prestained molecular weight markers (Amersham) were electrophoresed in parallel.

Immunoblot:

After 1 h incubation with a 1% BSA/99% TBS blocking solution at room temperature, the nitrocellulose was probed with primary PKC α monoclonal antibody (Transduction Labs, California) at a diluition of 1:1000 for 1 hr at room temperature. To detect bound primary antibody, blots were incubated for 1 hr at room temperature with a 1:5000 dilution of an alkaline phosphatase-conjugated anti-mouse secondary antibody (Sigma). The nitrocellulose was washed for 15 min x 3, then stained with a staining solution containing NBT/BCIP

for 10-15 sec, washed thoroughly with H_2O and let dry. Polyclonal antibodies against PKC isoforms were purchased from GIBCO-BRL (Grand Island, NY) and the monoclonal antibody against PKC was purchased from Upstate Biotechnology Inc.(Lake Placid, NY).

Measurement of Extracellular Release of H_2O_2

H_2O_2 release from adherent U87 cells into the overlying medium was determined using a procedure adapted from Ruch and co-workers (Ruch et al., 1983). Fluorescence was measured from horseradish peroxidase (HRP) catalyzed reaction of homovanillic acid (HVA) with H_2O_2. Briefly, cells were incubated with and without treatment in DMEM (without phenol red) containing 100 υM HVA, 5 U/ml HRP. This medium was collected after 4h, the pH was adjusted to 10.0 with 0.5 M glycine-NaOH buffer, and fluorescence was measured in a Perkin-Elmer fluorimeter (LS 50B), excitation and emission wavelengths of 321 nm and 421 nm, respectively. Data was analyzed by comparing average fluorescence of treatment groups to control samples. Results are presented as the mean + or - standard error of the means. Statistical difference between groups was assessed by ANOVA. If differences were found, Dunnett's post-hoc test was applied to determine significance. Values of $p<0.05$ were considered significant.

ATP measurements

Quantitative detection of ATP was performed as described in the ATP Bioluminescence Assay Kit CLS II (Boehringer Mannheim). Briefly, treated cells were washed twice in ice-cold PBS and were resuspended in a minimal volume of PBS. After addittion of nine volumes of of boiling extraction buffer (100 mM Tris-HCl, 4 mM EDTA, pH 7.75), samples were incubated for an additional 2 minutes at 100°C, and centrifuged at 10,000 x g for 60 seconds. Equal volumes of luciferase reagent and samples/standards were combined in a microtiter plate and assayed for bioluminescence using a Perkin Elmer LS 50B fluorimeter. Sample ATP concentrations were determined from a log-log plot of the standard curve data. Data was analyzed by comparing average ATP concentration of treatment groups to control samples. Results are presented as the mean + or - standard error of the means. Statistical difference between groups was assessed by ANOVA. If differences were found, Dunnett's post-hoc test was applied to determine significance. Values of $p<0.05$ were considered significant.

RESULTS

HIF-1 activation in U87 glioma cells is mediated through a decrease in cellular reactive oxygen species production.

Characterization of hypoxia inducible DNA binding activity in U87 glioma cells required control experiments to 1) ensure the specificity of the DNA probe for the inducible complex, and 2) to identify the inducible DNA binding protein through immunoreactivity. DFX is shown to induce DNA binding activity in U87 cells (Figure 4). All represented DNA/protein complexes are displaced by addition of increasing amounts of excess unlabelled WT probe to the binding reaction, but not by addition of increasing amounts of excess unlabelled MUT probe, containing a 3-base substitution in the HIF binding portion (Figure 4). These results indicate that the inducible and constitutive DNA binding proteins bind in a specific fashion with the 18 bp DNA probe containing the 8 bp HIF-1 consensus recognition element. The inducible DNA/protein binding complex was identified as a HIF-1 α containing complex as its mobility is retarded by the addition of a HIF-1 α monoclonal antibody to the binding reaction (Figure 5). Antigen specificity of this antibody has been previously

demonstrated (Huang et al., 1996). Addition of a monoclonal antibody against HIF-1 β to the binding reaction also results in a supershift of the inducible binding complex (data not shown). Specificity of the HIF-1 α antibody for the inducible protein in U87 cells is determined by the addition of antibody to control nuclear extract samples and probe alone (Figure 5).

Using gel shift analysis a four hour exposure of U87 cells to 1% oxygen is shown to induce HIF-1 DNA binding activity. The specificity of the hypoxia shifted HIF-1/DNA binding complex, often seen as a doublet (Figure 6), is demonstrated by the displacement of inducible binding activity by 200- fold excess WT unlabeled probe, but not by the MUT probe. As demonstrated in HEP3B cells (Semenza et al., 1992), pretreatment of U87 cells with CHX or Actinomycin D prevents hypoxic induction of HIF-1 DNA binding activity (Figure 6) demonstrating a requirement for ongoing protein and RNA synthesis. Treatment of control cells with these agents did not appear to be toxic, as there was no observable change in cellular morphology. Nor did treatment with these agents interfere with protein DNA binding activity (data not shown).

To determine whether HIF-1 activation by hypoxia occurs through ATP depletion, the effects of hypoxia with those of KCN were compared. While 4 hour exposure to either 1% oxygen or 2 mM KCN reduced cellular ATP levels to below 25% of baseline (Figure 7), KCN alone was unable to induce HIF-1 binding activity under normoxia (Figure 7). A prominent activation of HIF-1 by hypoxia was observed in the presence or absence of KCN, suggesting that U87 cells contain an oxygen sensing mechanism which is independent of changes in cellular ATP levels. Furthermore, simultaneous exposure of U87 cells to 1% oxygen and 10% CO prevented HIF-1 activation supporting the involvement of a hemoprotein in glial hypoxic signaling which directly senses oxygen or an oxygen metabolite.

In addition to lowering cellular ATP levels, hypoxia also results in the lowering of reactive oxygen metabolites such as superoxide and hydrogen peroxide. To determine whether the hypoxia signaling mechanism in U87 cells was responsive to a decline in ROS, nonspecific antioxidants and DFX were tested in their ability to induce HIF-1 under normoxic conditions (Figure 8). DFX activated HIF-1 in an H_2O_2 and iron reversible manner. Other antioxidants, diphenylphenylenediamine (DPPD) and dithionite also activated

HIF-1 (Figure 8). Addition of oxidants N-ethylmaleimide and H_2O_2 to the vitro binding assay had no effect on HIF-1 binding properties (data not shown).

Addition of 1mM H_2O_2 to cells during hypoxia prevented HIF-1 activation (Figure 9). In order to avoid possible toxic actions of H_2O_2 addition, catalase was added to the media to lower the baseline levels of H_2O_2 in normoxic cultures. H_2O_2 is membrane permeable and is effectively and specifically depleted by extracellular catalase addition (Fandrey et al., 1994, Kroll and Czyzyk-Krzeska, 1998). Catalase activated HIF-1 binding activity (Figure 9).

To identify the source of normoxic H_2O_2 production which regulates HIF-1 in U87 cells, several pharmacological inhibitors of ROS production were used to lower H_2O_2 and to stimulate HIF-1 DNA binding activity under normoxia. Basal production of H_2O_2 by U87 cells was lowered by hypoxia and catalase (Figure 10). Allopurinol, SKF-525a, and ibuprofen, inhibitors of xanthine oxidase, cytochrome p450, and cyclooxygenase, respectively had no effect on basal H_2O_2 levels over the 4 hour incubation. Since the mitochondria is a major consumer of cellular oxygen and a supplier of cellular ROS (Herrero and Barja, 1997), inhibitors of

mitochondrial ROS production were tested on their ability to decrease basally produced H_2O_2 in U87 glioma cells. Thenoyltrifluoroacetone (TTFA), an inhibitor of mitochondria ET complex II lowered normoxic H_2O_2 production in U87 cells (Figure 10) and was also found to dose dependently activate HIF-1 under normoxia (Figure 11). Addition of 250nM Rotenone, another inhibitor of mitochondrial (ROS) production, to normoxic cultures for 4 hours also activated HIF-1 (Figure 11). The effects of TTFA were reversed by 1mM H_2O_2 , and the TTFA induced binding complex was shown to contain HIF-1 α by supershift analysis (Figure 12).

Figure 4: *Specificity of the HIF-1 DNA probe is determined by competition analysis.* Inducible binding activity is displaced by excess unlabelled wild type (WT) probe and not by excess unlabelled mutant (MUT) probe. Procedure and probe sequences are described in methods section. From left to right: Control; desferrioxamine (DFX); nuclear extract from DFX treated cells + 150X WT probe; + 300X WT; + 600X WT; + 150X MUT; +300X MUT; +600X MUT. (C= constitutive binding)

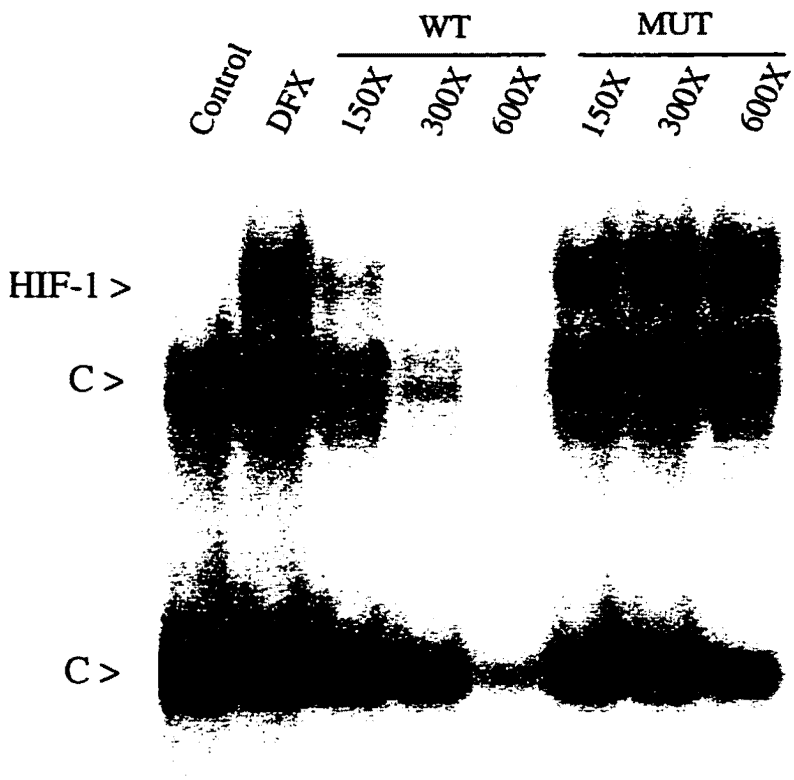

WT
MUT
Control
DFX
150X
300X
600X
150X
300X
600X
HIF-1 >
C >
C >

Figure 5: *HIF-1 α antibody immunoreactivity is specific for the inducible DNA binding protein.* Supershift procedure and antibody information is described in methods section. From left to right: DFX; DFX + 1:20 dilution of HIF-1 α antibody (Ab); Probe + 1:100, 1:50, 1:20 dilution of HIF-1 α antibody; Control (normoxia) + 1:20 dilution of HIF-1 α antibody; Probe + 1:100, 1:50, 1:20 dilution of HIF-1 α antibody; Free probe; Probe + 1:20 dilution of HIF-1 α antibody in binding buffer not containing calf thymus DNA, nonspecific inhibitor (NSI). (SS = supershift; C= constitutive binding)

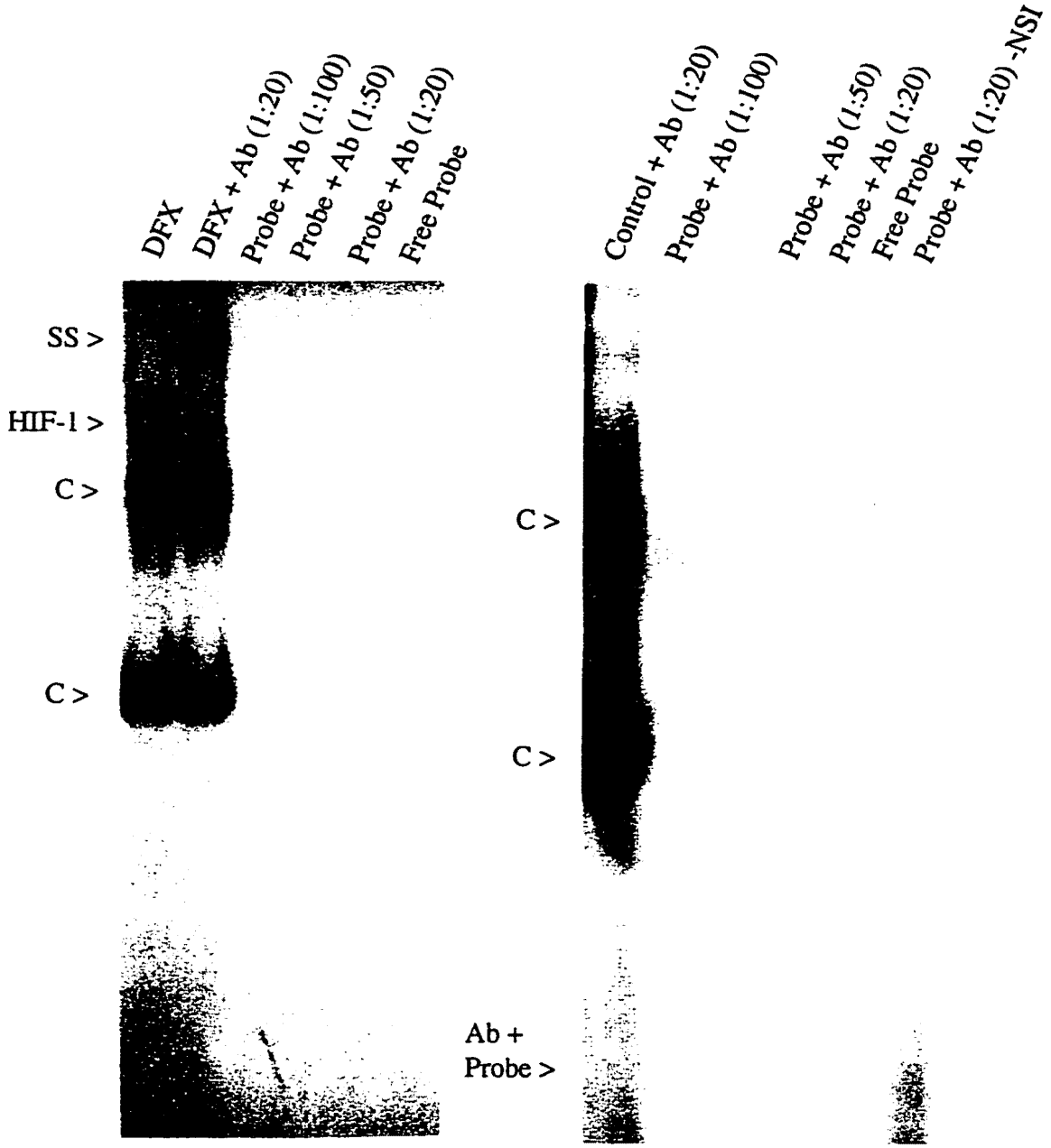

DFX
DFX + Ab (1:20)
Probe + Ab (1:100)
Probe + Ab (1:50)
Probe + Ab (1:20)
Free Probe
Control + Ab (1:20)
Probe + Ab (1:100)
Probe + Ab (1:50)
Probe + Ab (1:20)
Free Probe
Probe + Ab (1:20) -NSI
SS >
HIF-1 >
C >
C >
C >
C >
Ab +
Probe >

Figure 6: *Hypoxia-inducible binding activity is not induced by ATP depletion.* Cells were treated for 4 hours, nuclear extracts and gelshift analysis were performed as described in methods. Treatments or conditions from left to right were: Normoxia (21% O_2), hypoxia (1% O_2), hypoxic extract with 200-fold molar excess of wild type unlabeled consensus sequence, 200-fold molar excess unlabeled mutant consensus sequence, hypoxia plus 100uM cycloheximide (CHX), hypoxia plus 10ug/ml actinomycin D, normoxia plus 2mM potassium cyanide (KCN), hypoxia plus 2mM KCN, or hypoxia plus 10% carbon monoxide (CO). Similar results were observed in replicates of these treatments (n=3). (C = Constitutive binding activity; WT=wild type; MUT= mutant)

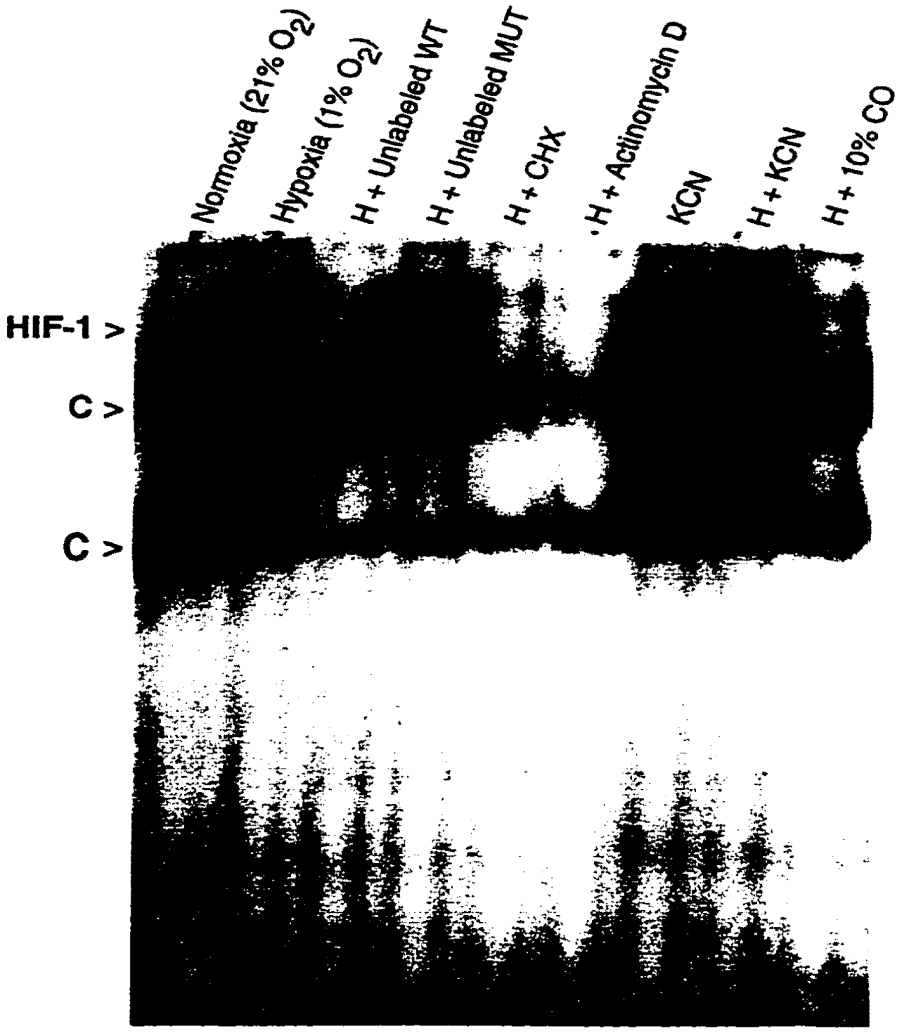

Nomoxia (21% O2)
Hypoxia (1% O2)
H + Unlabeled WT
H + Unlabeled MUT
H + CHX
H + Actinomycin D
KCN
H + KCN
H + 10% CO
HIF-1 >
C >
C >

Figure 7: *ATP depletion by hypoxia and cyanide.* Intracellular ATP concentrations were determined as described in methods. U87 cells were exposed to 21% oxygen, 1% oxygen, 2mM potassium cyanide (KCN), or 2mM potassium cyanide (KCN) + 1% oxygen for 4 hours. Values are expressed as means of 3 experiments +/- S.E.M. Data was analyzed using analysis of variance (ANOVA), Dunnett's post-hoc test for significance (* = $p<0.001$)

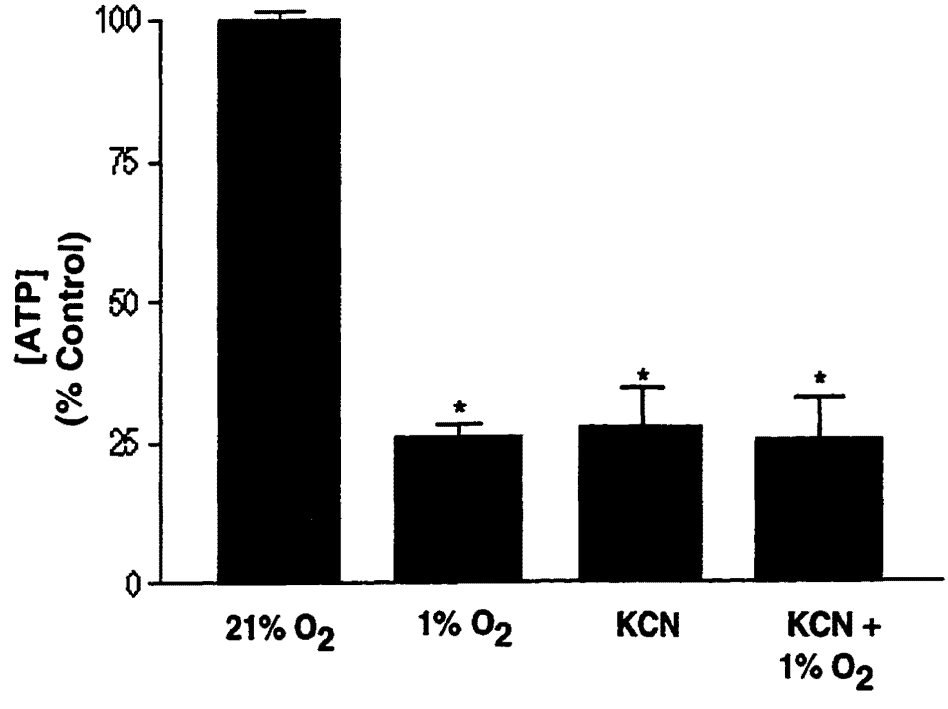

100
75
50
25
0
[ATP]
(% Control)
*
*
*
21% O_2
1% O_2
KCN
KCN +
1% O_2

Figure 8: *HIF-1 activation by nonspecific antioxidants.* Four hour treatments or conditions from left to right were: Normoxia, hypoxia, 130uM Desferrioxamine (DFX); 130uM DFX + 260uM ferrous chloride (Fe), DFX + 1mM H_2O_2 , hypoxia + 1mM H_2O_2 , 1uM Dithionite (DTH), 1uM Curcumin (DFM), 1uM diphenylphenylenediamine (DPPD) Similar results were observed in replicates of these treatments (n=4 for each, except n=2 for antioxidants, n=5 for hypoxia + H2O2 and n=8 for DFX)(C = Constitutive binding activity)

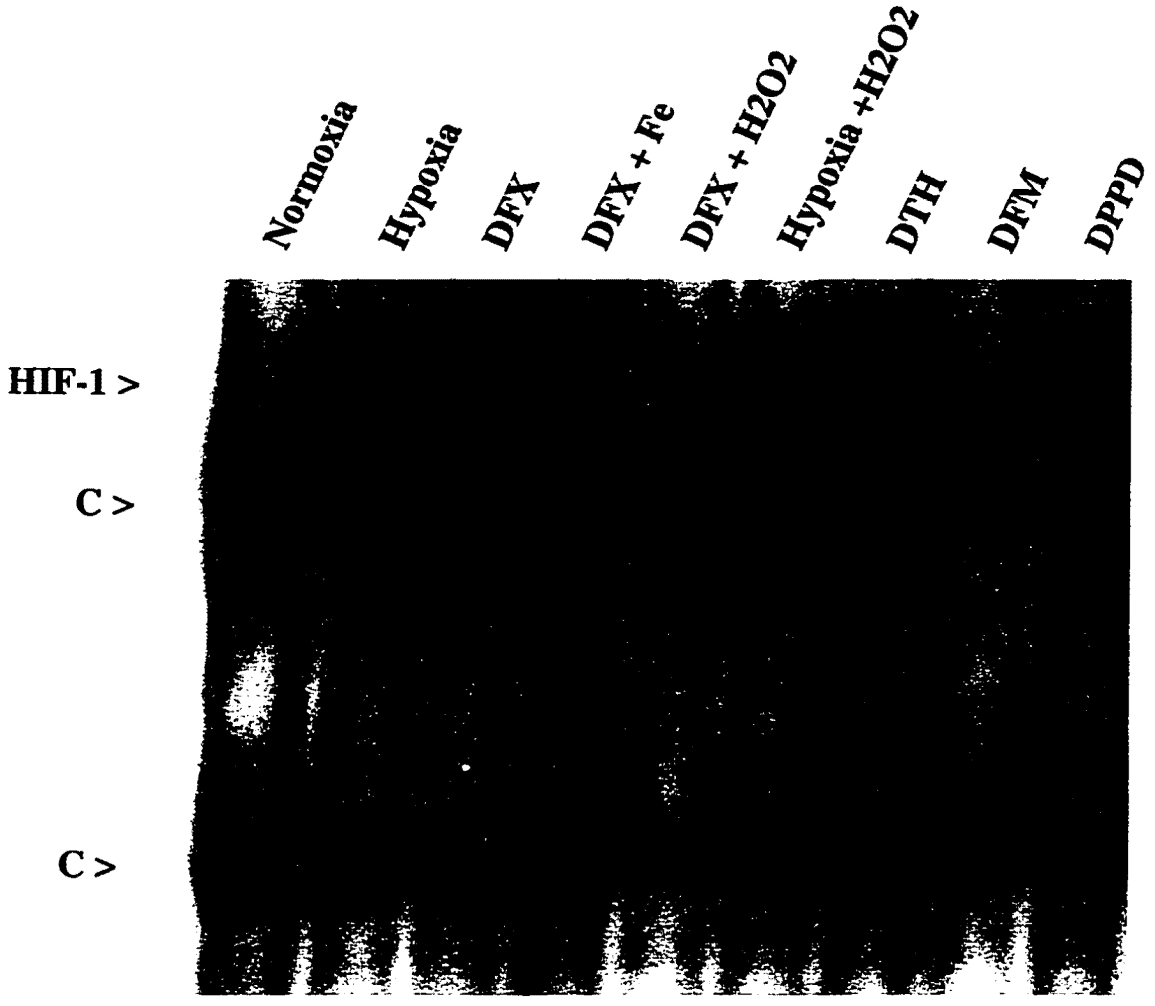

Normoxia
Hypoxia
DFX
DFX + Fe
DFX + H2O2
Hypoxia +H2O2
DTH
DFM
DPPD
HIF-1 >
C >
C >

Figure 9: *Activation of HIF-1 binding activity in U87 cells by lowering cellular H2O2 levels.* Cells were exposed to, normoxia, hypoxia, hypoxia + 1mM H2O2, or 5,000U/mL catalase for 4h and analyzed for HIF-1 activation as described in methods. Similar results were observed in replicates of these treatments (n=5).(C = Constitutive binding activity)

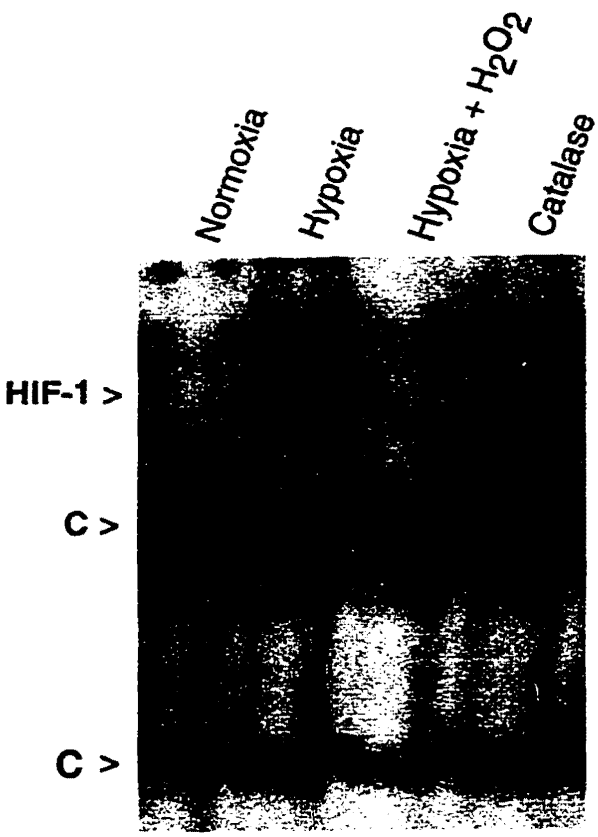

Normoxia
Hypoxia
Hypoxia + H_2O_2
Catalase
HIF-1 >
C >
C >

Figure 10: *Pharmacological determination of major cellular source of normoxically derived H2O2 in U87 cells.* Peroxidase catalyzed dimerization of HVA was used to assay for cellular H_2O_2 , as described in methods. Treatments (4h) were, from left to right: 21% oxygen, 1% oxygen, 5000U/ml catalase, 200uM Allopurinol, 200uM Ibuprofen, 20uM SKF525A, 50uM TTFA, or 2mM potassium cyanide (KCN). Values are expressed as means of 4 experiments +/- S.E.M. Data was analyzed using analysis of variance (ANOVA), Dunnett's post-hoc test for significance (* = $p<0.001$).

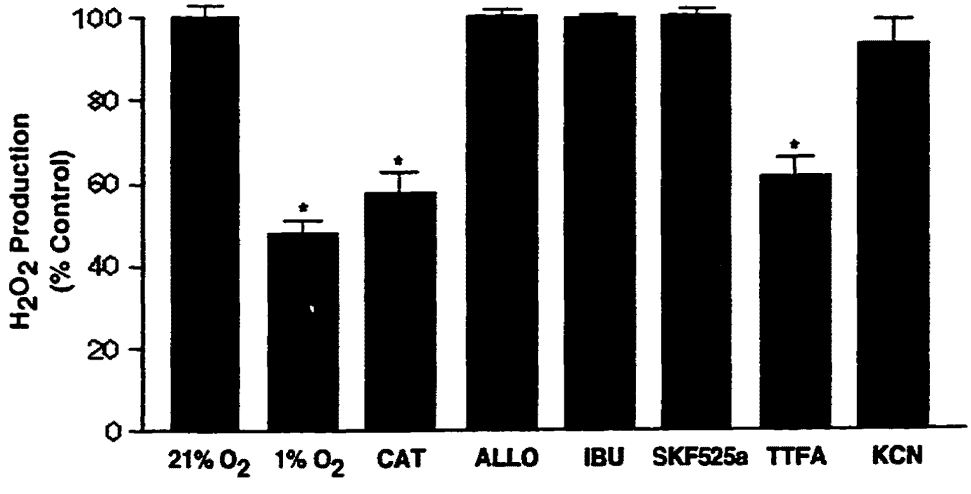

H_2O_2 Production
(% Control)
100
80
60
40
20
0
*
*
*
21% O_2
1% O_2
CAT
ALLO
IBU
SKF525a
TTFA
KCN

Figure 11: *Dose dependent activation of HIF-1 binding activity in normoxic U87 cells by treatment with TTFA and by treatment with rotenone.* Normoxic cultures were treated with either 10uM, or 50uM TTFA for 4h, or 250nM Rotenone (ROT) for 4 hours and analyzed for HIF-1 activation as described in methods. Similar results were observed in replicates of these treatments (n=5 for TTFA; n=3 for Rotenone).(C = Constitutive binding activity)

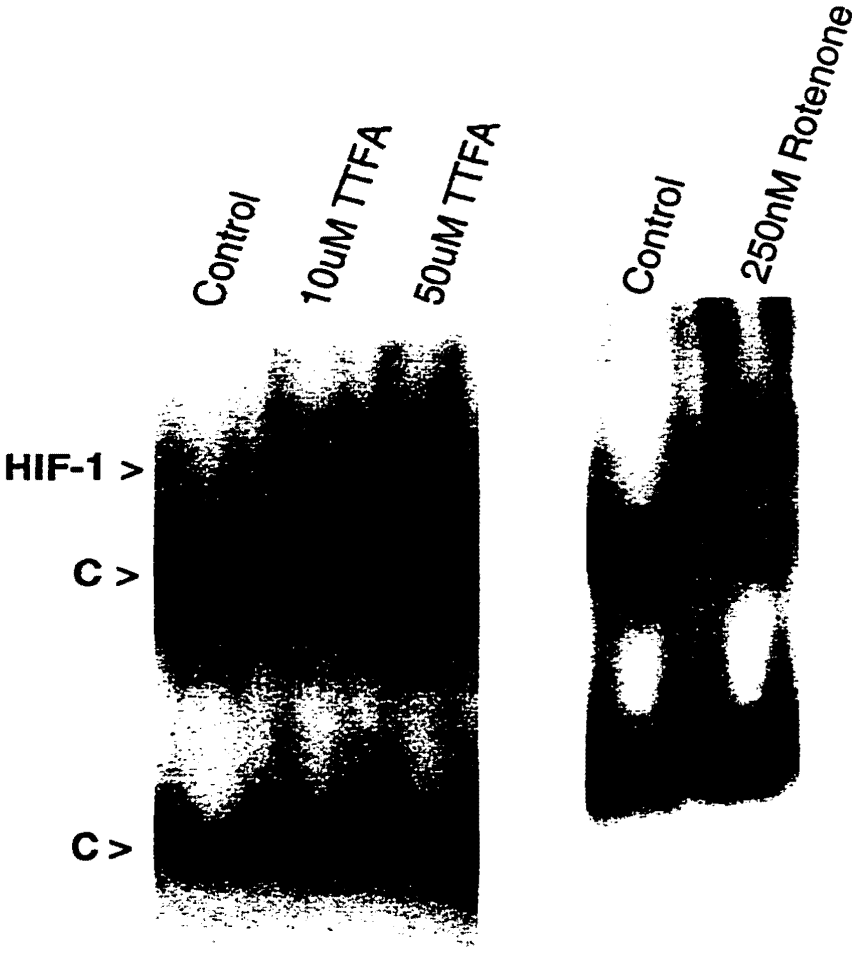
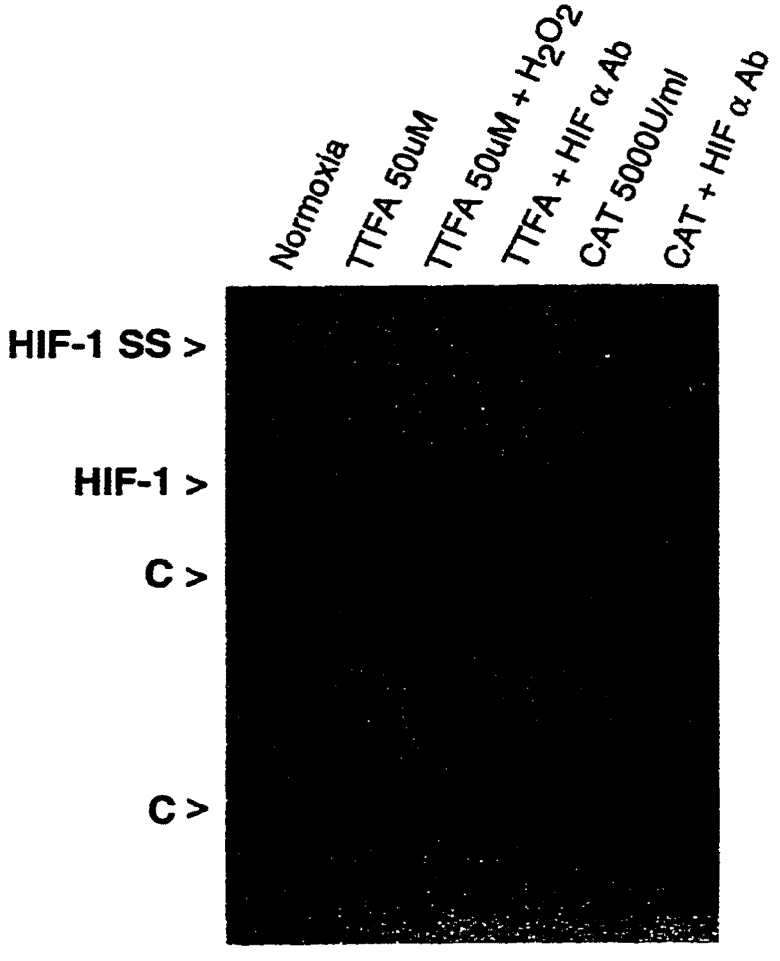

Control
10uM TTFA
50uM TTFA
Control
250nM Rotenone
HIF-1 >
C >
C >

Figure 12: *Inhibition of TTFA induced HIF-1 activation by H2O2. Specificity of HIF-1 binding is determined by supershift analysis in TTFA and catalase extracts.* From left to right: 21% oxygen alone, + 50uM TTFA, + 50uM TTFA + 1mM H2O2, 50uM TTFA treated nuclear extract + 1:10 dilution of anti-HIF-1 α antibody (1uL OZ15 + 1uL OZ12), 5000U/ml catalase (CAT) treated nuclear extract + 1:10 dilution of anti-HIF-1 α antibody. Similar results were observed in replicates of these treatments (n=5 for TTFA, n=3 for TTFA + H2O2, n=5 for catalase). (HIFSS = Supershift; C = Constitutive binding activity)


Normoxia
TTFA 50uM
TTFA 50uM + H_2O_2
TTFA + HIF α Ab
CAT 5000U/ml
CAT + HIF α Ab
HIF-1 SS >
HIF-1 >
C >
C >

HIF-1 activation in U87 glioma cells involves protein kinase C enzymatic activity.

HIF-1 α is a phosphoprotein and dephosphorylation of hypoxic nuclear extracts from Hep3B hepatoma cells has been shown to abrogate HIF-1 DNA binding (Wang and Semenza, 1993). Using hypoxic nuclear extract of U87 cells, hypoxia-activated HIF-1 DNA binding activity is reversed by addition of phosphatases to the binding reaction (Figure 13). To avoid a nonspecific effect of protein phosphatases on the HIF-1 DNA binding reaction agarose immobilized phosphatase was used in the treatment. Pretreatment of hypoxic nuclear extracts with immobilized alkaline phosphatase followed by its removal by centrifugation prior to performing the binding reaction with the supernatant is very effective in reversing the hypoxia-activated DNA binding activity without any significant effect on constitutive binding activity (Figure 13). These results demonstrate that activation of HIF-1 DNA binding activity by hypoxia requires phosphorylation of nuclear proteins by unknown kinases.

Exposure of U87 cells to the general serine threonine protein kinase inhibitor 2-aminopurine prevents the activation of HIF-1 under hypoxic conditions (Figure 14).

Specific inhibitors of major classes of protein kinases failed to block the induction of HIF-1 by hypoxia (Figure 15). Tyrosine kinase inhibitor (herbimycin A), protein kinase A inhibitor (KT5720), protein kinase G inhibitor (KT5823), and protein kinase C inhibitor (bisindolmaleimide II) did not inhibit HIF-1 induction (Figure 15). Due to a decrease in inducible and constitutive binding with 100nM bisindolmaleimide treatment, the effect of this treatment was determined to be nonspecific and potentially toxic. To further explore the role of PKC in hypoxic signaling, chelerythrine chloride, another selective PKC inhibitor showed potent antagonism against hypoxic activation of HIF-1 (Figure 16). This initial implication of PKC involvement in hypoxic signaling was confirmed by using the direct PKC activator phorbol myristate acetate (PMA). 100nM PMA potently stimulated HIF-1 DNA binding activity within two hours under normoxia, authentication of HIF-1 transcription factor was determined by supershift assay (Figure 17).

This effect could also be inhibited by chelerythrine chloride (Figure 18). Catalase activation of HIF-1 was also blocked by

chelerythrine chloride, suggesting that PKC activation of HIF-1 is downstream from ROS mediated signaling (Figure 18). These observations strongly implicate the involvement of a PKC isoenzyme in the hypoxia signaling pathway activating HIF-1 in U87 cells. Glioma cells are known to predominantly express the PKC α isoform (Misra-Press et al., 1992), and using western blot analysis, the presence of PKC α in U87 astrocytoma cells was demonstrated. While particulate fractions of normoxic U87 cells show baseline levels (55% of total) of PKC α, there is a prominent translocation of PKC α from the cytosolic fraction to the particulate fraction after exposure to 1% O_2 (88% of total), or catalase (78% of total), for 2.5 hours (Figure 19). Particulate localization of PKC α and γ isoforms under normoxic conditions has been demonstrated in human glioma cells (Mira-Press, 1992). The hypoxic translocation of PKC α is blocked by exogenously added H_2O_2 and 10% CO supporting the idea that these agents act at a site upstream from this component of the oxygen signaling pathway leading to HIF-1 induction (Figure 20). Collectively, these findings suggest that PKC activation is a critical component of the U87 hypoxic signaling leading to HIF-1 activation.

The major stimulators of HIF-1 DNA binding activity were also tested on their ability to increase HIF-1 α nuclear protein levels, a measure that may not coincide with HIF-1 DNA binding activity. Treatment of cells with hypoxia, catalase, or PMA for 4 hours results in an increase in HIF-1 α nuclear protein as revealed by western blot analysis using a HIF-1 α monoclonal antibody (Figure 21). Densitometric analysis of the immunoreactive band corresponding to the 97 kD protein standard (approximate size of HIF-1 α) reveals a 3.8 fold induction of HIF-1 α by hypoxia, a 1.6 fold increase by catalase, and a 2.3 fold increase by PMA(Figure 21). These measurements are taken from lanes in which 50 υg of nuclear extract was loaded.

Much of the foundation in our knowledge of hypoxic signaling comes from the studies using HEP3B cells. To test the potential involvement of modulation of ROS and PKC on HIF-1 activation in these cells, stimulators of HIF-1 activation in U87 cells were used (Figure 22). As others have previously demonstrated DFX is a potent stimulator of HIF-1 binding in HEP3B cells (Semenza et al., 1992). Treatment of cells with

catalase at a dose similar to that used in U87 cells did not activate HIF-1. However, liver cells are known to have high levels of endogenous catalase, relative to glia (Halliwell, 1983). Higher dosages of exogenous catalase may be required to elicit the same effects. TTFA treatment and PMA treatment are effective in inducing HIF-1. Also, 4-Aminopyridine (4-AP), a selective antagonist of the ROS sensitive potassium channel, induces HIF-1 (Figure 22). As presented in the discussion section, this potassium channel may play a major universal role in hypoxic signaling, and may provide a link between ROS mediated signaling and PKC enzyme activity.

Figure 13: *Phosphatase treatment of hypoxic nuclear extracts extinguishes HIF-1/ DNA binding.* EMSA was performed using nuclear extract from cells incubated in hypoxia for 4 h. From left to right: hypoxic extract without sodium vanadate (VAN) added to the binding reaction; or, after treatment of nuclear extract from hypoxic U87 cells with either 0.5 U alkaline phosphatase; 0.5 U potato acid phosphatase (PAP); 1.67 U immobilized alkaline phosphatase (IAP); 0.66 U IAP; or, 0.33 U IAP. Immobilized alkaline phosphatase treatment was performed as described in methods. This work confirms findings previously reported in HEP3B cells (Wang et al., 1993) (n=1) (C=Constitutive binding activity)

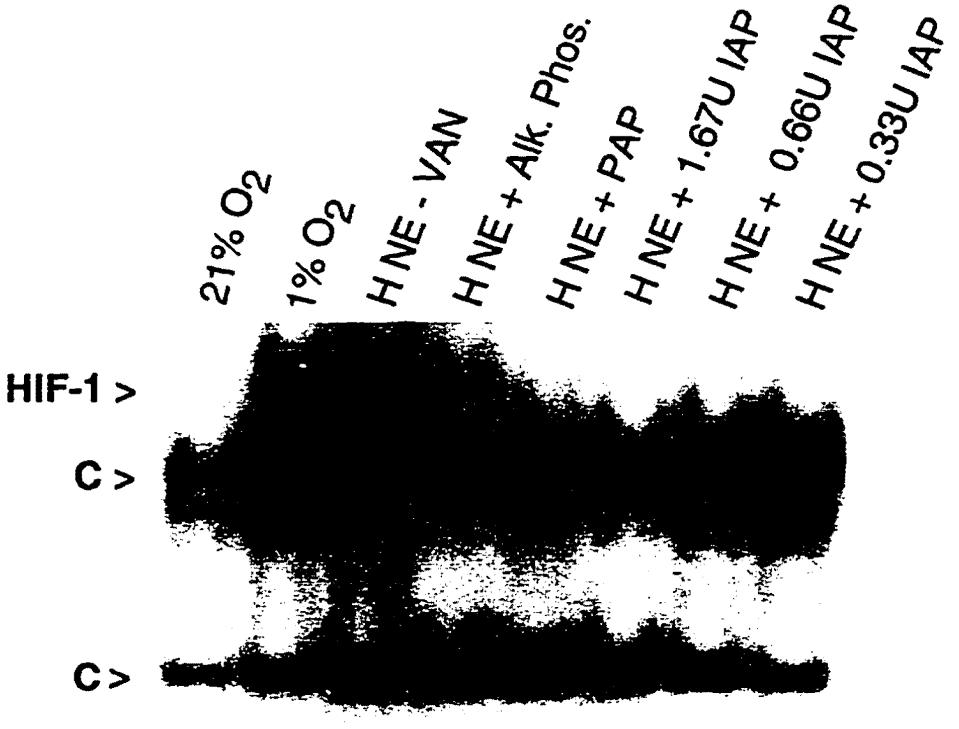

21% O2
1% O2
H NE - VAN
H NE + Alk. Phos.
H NE + PAP
H NE + 1.67U IAP
H NE + 0.66U IAP
H NE + 0.33U IAP
HIF-1 >
C >
C >

Figure 14: *Hypoxic induction of HIF-1 binding activity is blocked by 2-aminopurine (2-AP).* EMSA was performed using nuclear extract which were incubated in normoxia (lane 1); hypoxia (1% oxygen) for 4 h (lane 2); or, hypoxia plus 5mM 2-AP for 4h. This data confirms previously reported work in HEP3B cells (Wang et al., 1993) (n=2)(C=constitutive binding activity)

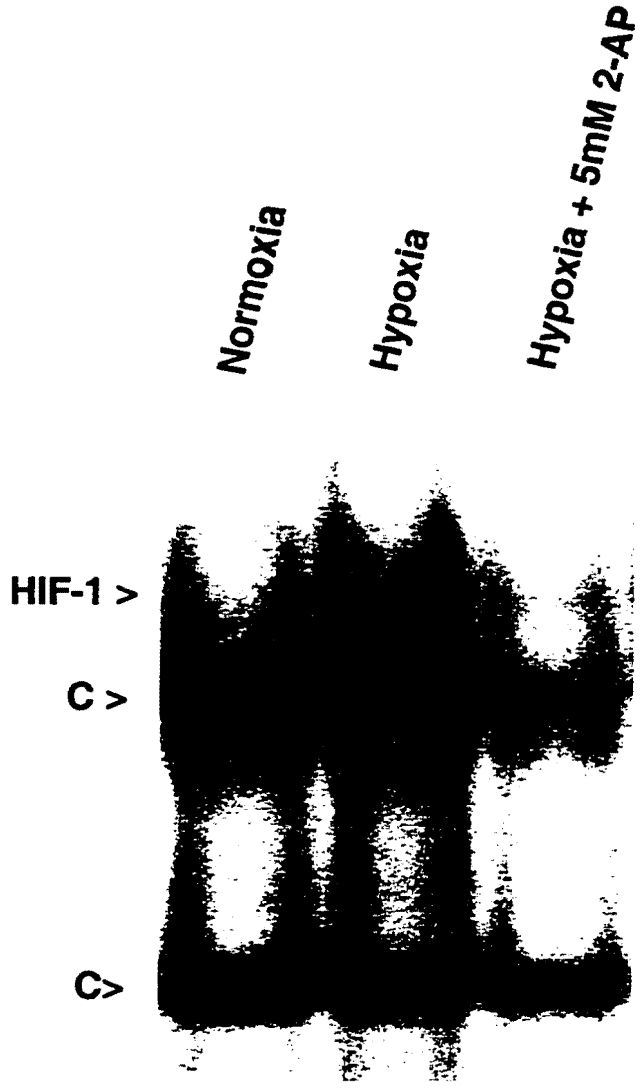

Normoxia
Hypoxia
Hypoxia + 5mM 2-AP
HIF-1 >
C >
C>

Figure 15: *Hypoxic induction of HIF-1 binding activity is not blocked by specific kinase inhibitors.* EMSA was performed using nuclear extract from U87 glioma cells which were treated (from left to right) in normoxia (21% oxygen); hypoxia (1% oxygen) for 4 h; or, hypoxia plus 1uM herbimycin A (Herb A); hypoxia plus 5uM herbimycin A; hypoxia plus 0.5uM KT5720; hypoxia plus 1uM KT5720; hypoxia plus 0.5uM KT5823; hypoxia plus 1uM KT5823; hypoxia plus 50nM bisindolmaleimide II; hypoxia plus 100nM bisindolmaleimide II (BIS II). Similar results were observed in replicates of these treatments (n=2 for higher dosages of inhibitors, n=4 for bisindolmaleimide) (C=Constitutive binding activity)

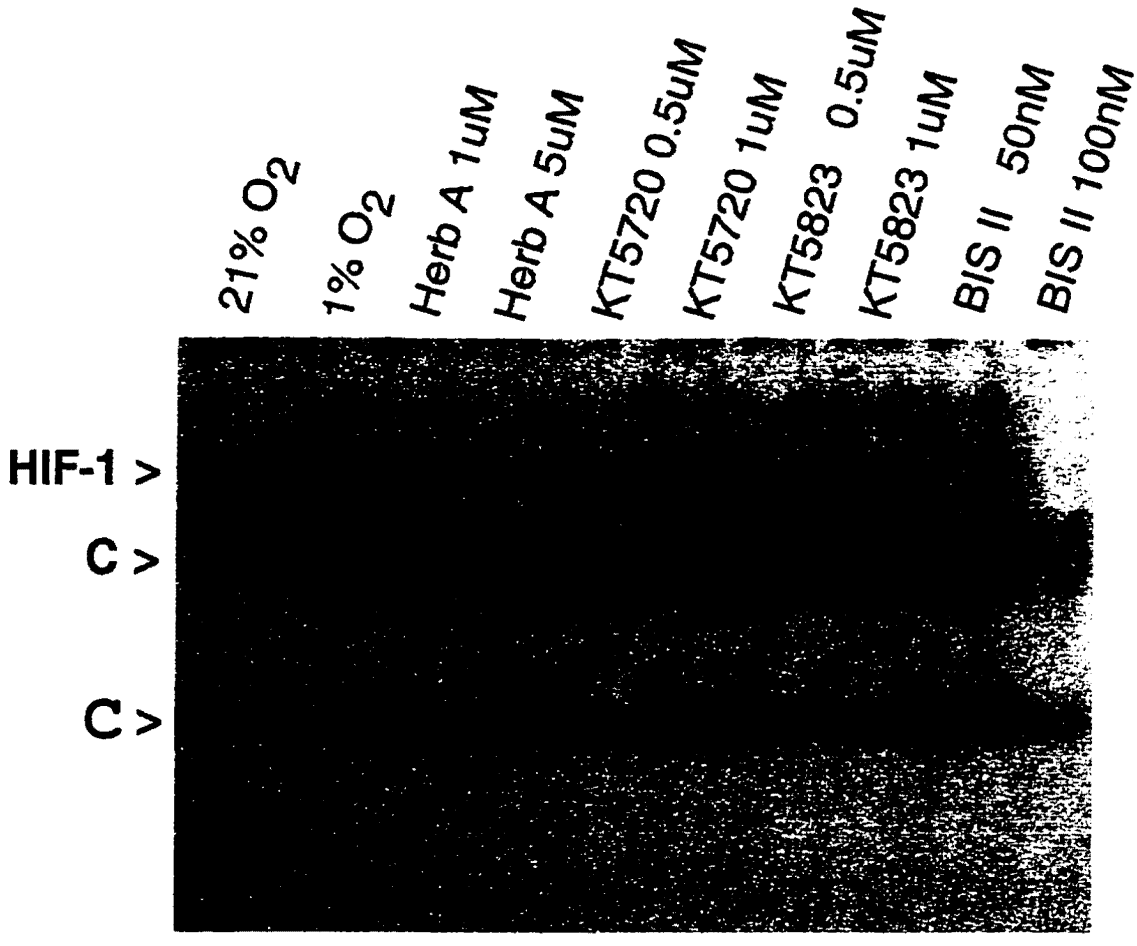

21% O2
1% O2
Herb A 1uM
Herb A 5uM
KT5720 0.5uM
KT5720 1uM
KT5823 0.5uM
KT5823 1uM
BIS II 50nM
BIS II 100nM
HIF-1 >
C >
C >

Figure 16: *Hypoxic induction of HIF-1 binding activity is blocked by chelerythrine chloride in a dose dependent manner.* EMSA was performed using nuclear extracts from U87 glioma cells which were incubated under normoxia (21% O_2); hypoxia (1% O_2) for 2.5 h; hypoxia plus 1uM chelerythrine chloride (CH); or hypoxia plus 10uM CH. Similar results were observed in replicates of these treatments (n=3) (C=Constitutive binding activity)

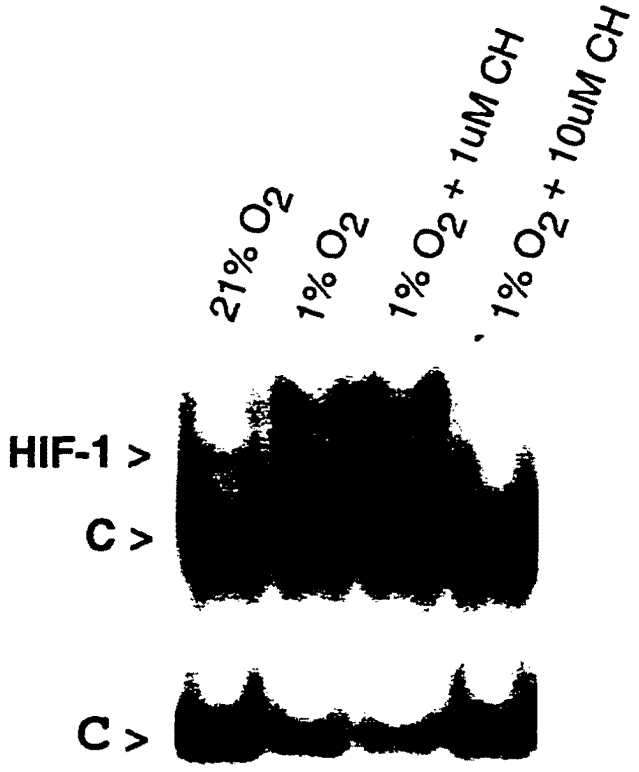

21% O2
1% O2
1% O2 + 1uM CH
1% O2 + 10uM CH
HIF-1 >
C >
C >

Figure 17: *HIF-1 activity is stimulated by phorbol myristate acetate (PMA)*. From left to right, normoxia (Control), 100nM PMA (2.5hr), supershift with PMA treated extracts + 2ul HIF-1 a Antibody. Similar results were observed in replicates of these treatments (n=3 for PMA).(C=Constitutive binding activity) **Note**: Lanes between normoxia and PMA samples were excised so that a comparison could be made between control and treated cells.

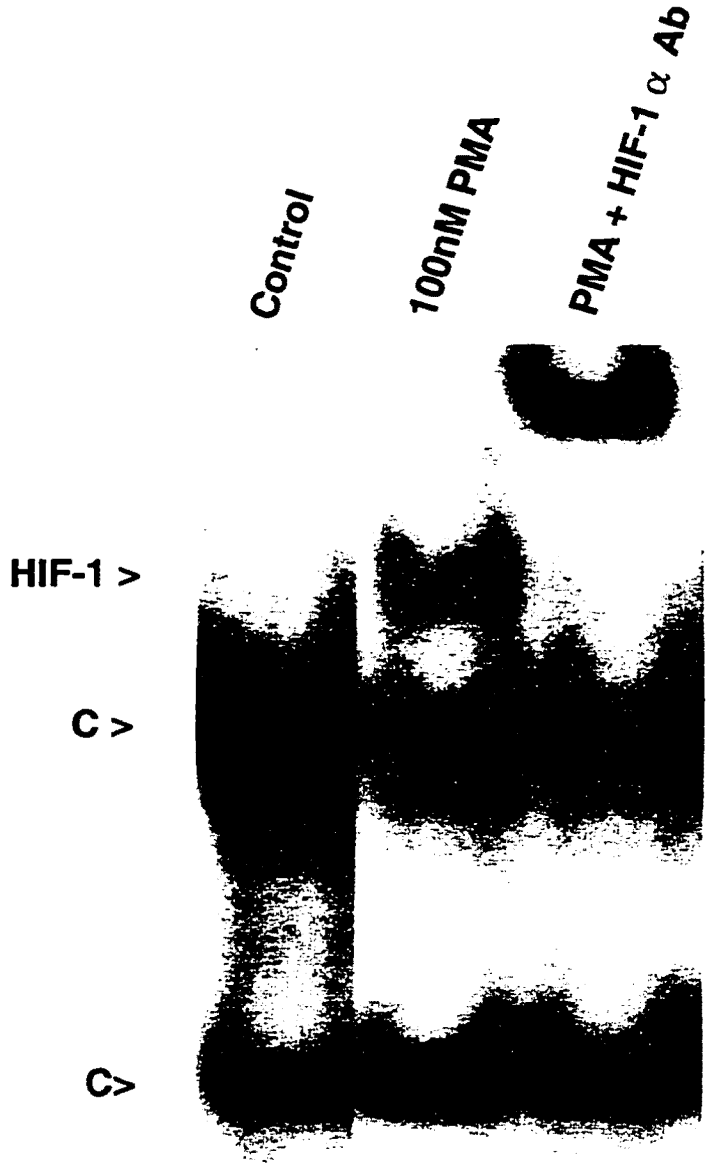

Control
100nM PMA
PMA + HIF-1 α Ab
HIF-1 >
C >
C>

Figure 18: *HIF-1 binding activity induced under normoxic conditions by phorbol myristate acetate (PMA) and catalase (CAT) is blocked by coincubation with chelerythrine chloride.* EMSA was performed using nuclear extracts from U87 glioma cells which were incubated under normoxia (21% O_2); Normoxia plus 100nM phorbol myristate acetate (PMA) for 2.5 h; PMA plus 10uM chelerythrine chloride (CH); Normoxia plus 5000U/ml catalase (CAT) for 2.5 h; or, catalase plus 10uM CH. Similar results were observed in replicates of these treatments (n=3 for PMA, n=5 for CAT, n=2 for inhibitors). (C=Constitutive binding activity)

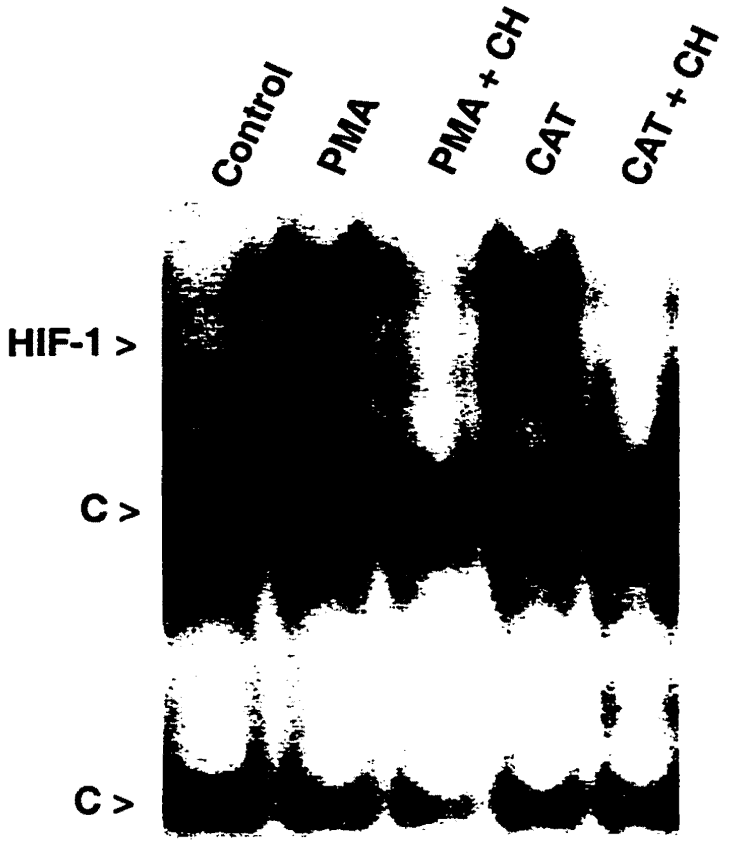

Control
PMA
PMA + CH
CAT
CAT + CH
HIF-1 >
C >
C >

Figure 19: *PKC α translocation to the particulate cellular fraction in U87 cells is triggered by hypoxia (1% O2) and 5000U/ml catalase (CAT) treatment for 2.5 hours.* Procedure was performed as described in Methods. Similar results were observed in replicates of these treatments (n=3, n=2 for CAT)(C= Cytosolic fraction; P= Particulate fraction)

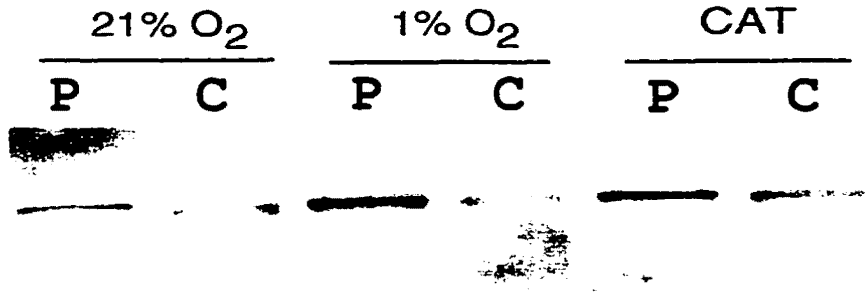

21% O_2
1% O_2
CAT
P
C
P
C
P
C

Figure 20: *Inhibition of hypoxia-mediated translocation of PKC α by H_2O_2 and CO in U87 cells.* Cells were treated with wither normoxia (21% oxygen); hypoxia (1% oxygen); hypoxia + 1 mM hydrogen peroxide (H2O2); or hypoxia + 10% carbon monoxide (CO) for 4 hours. PKC translocation experiments were performed as described in methods. Similar results were observed in replicates of these treatments (n=3, n=2 for hypoxia + H2O2 and hypoxia + CO).(C= Cytosolic fraction; P= Particulate fraction)

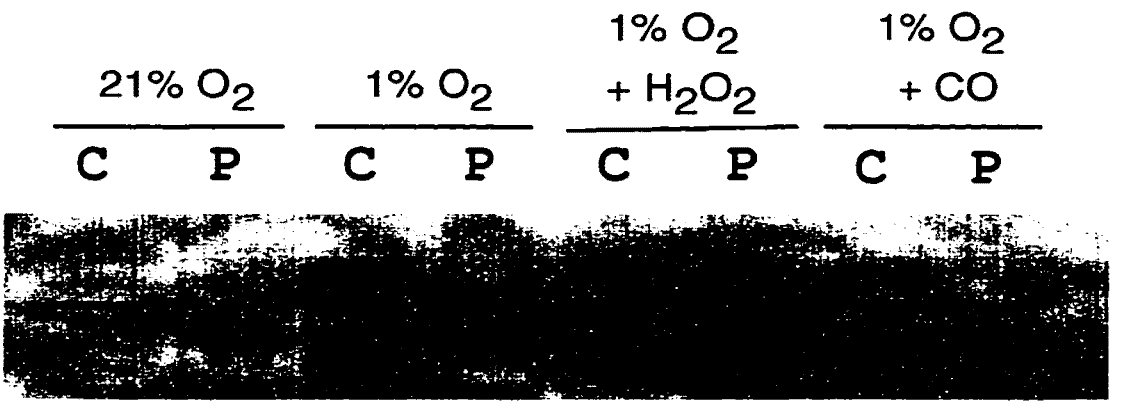
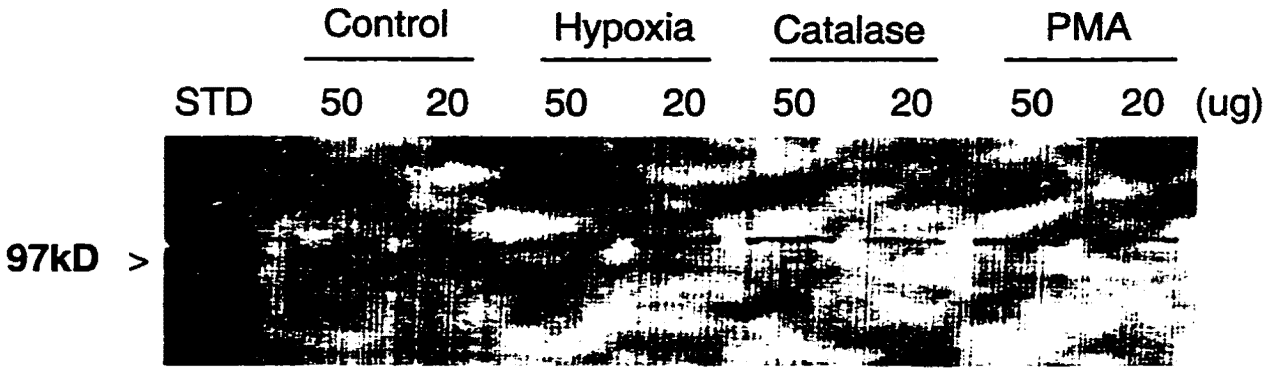

21% O_2
1% O_2
1% O_2 + H_2O_2
1% O_2 + CO
C P C P C P C P

Figure 21: *HIF-1 α immunoreactivity in U87 cell nuclear extracts is increased by treatment with hypoxia, catalase, and PMA.* Western blot procedures and methods for data analysis are described in the methods section. HIF-1 α immunoreactivity for either 50 or 20 ug of nuclear extract from control, hypoxia (1% O2), 5,000 U/ml catalase, or 100 nM PMA treated cells is represented. Densitometric analysis was performed using the band representative of 50 ug of nuclear extract for each treatment. Relative magnitude of treatment induced increase in immunoreactivity is represented in the histogram. Statistical analysis was not performed on this data due to the limited number of replicates for each treatment (Control, n=2, hypoxia, n=2; catalase, n=1; PMA, n=1)


Control
Hypoxia
Catalase
PMA
STD
50
20
50
20
50
20
50
20
(ug)
97kD >

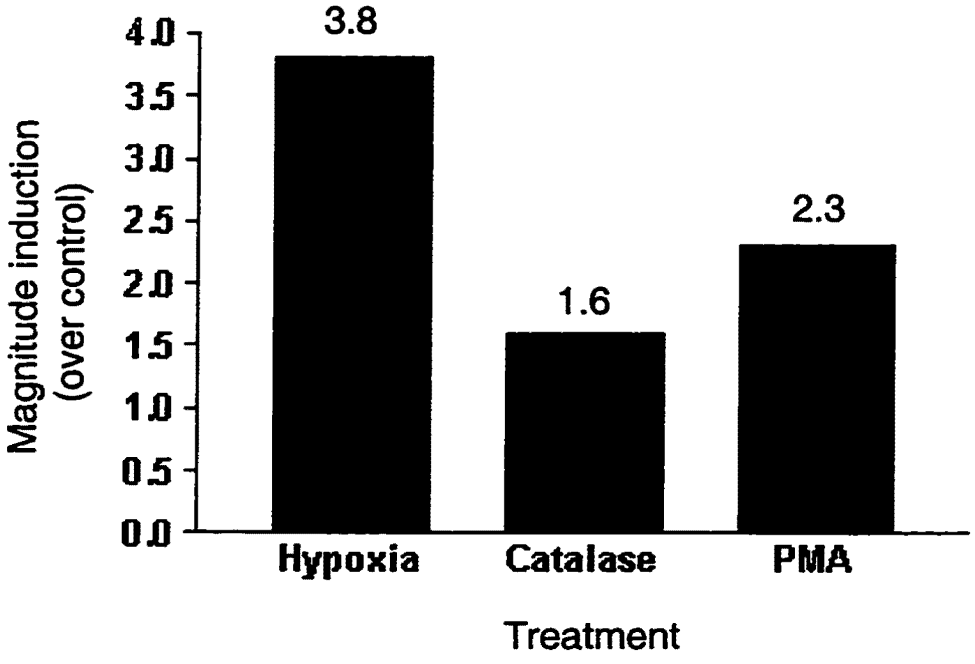
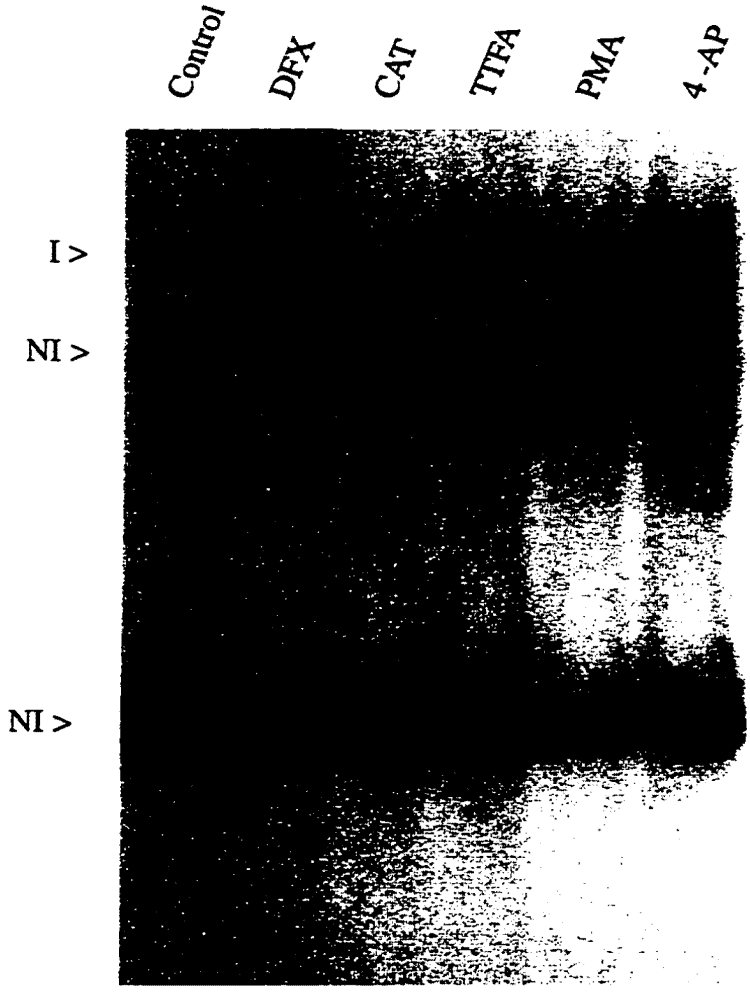

Magnitude induction
(over control)
4.0
3.5
3.0
2.5
2.0
1.5
1.0
0.5
0.0
3.8
1.6
2.3
Hypoxia
Catalase
PMA
Treatment

Figure 22: *Stimulators of the hypoxia signaling pathway in U87 cells also stimulate HIF-1 binding activity in HEP3B cells.* HEP3B cell treatment and nuclear extract were prepared using the same methods as described for U87 cells. Gel shift analysis was performed using samples from cells treated for 4 hours with the following (from left to right): Control; 130uM DFX; 5000 U/ml catalase; 100uM TTFA; 100nM PMA (2 hr. treatment); and 4-aminopyridine (4-AP). The identity of the inducible DNA binding protein was not determined using supershift analysis. The specificity of the DNA probe was not determined using competition analysis (n=1) (I = inducible; NI = non-inducible)


Control
DFX
CAT
TTFA
PMA
4-AP
I >
NI >
NI >

DISCUSSION

The major finding of this study is that hypoxic signaling mechanisms in human glioma cells respond to changes in cellular H_2O_2 levels. This is supported by the sensitivity of hypoxic signaling to changes in oxygen levels but not to changes in cellular ATP levels. H_2O_2 is freely diffusible across cellular membranes and intracellular H_2O_2 levels can thus be changed by manipulating the culture medium. The addition of exogenous H_2O_2 to the culture medium prevented hypoxic activation of HIF-1 without major effects on cell morphology over 4 hours. While this approach has been employed previously to study the role of H_2O_2 in cell signaling (Barrington et al., 1997) it may have toxic side effects. Catalase, an H_2O_2 metabolizing enzyme which cannot enter cells but can effectively "sponge" H_2O_2 from the cytoplasm also activated HIF-1 under normoxia. Collectively, these results suggest that baseline H_2O_2 production under normoxia may keep a hypoxia signaling pathway constituitively inhibited. Hypoxia, by lowering cellular H_2O_2 levels may thus disinhibit this pathway.

Mitochondrial ETC activity represents the largest source of basal ROS production in many cell types (Forman and Boveris, 1982). Within the mitochondrial ETC, the major sites of ROS production have been identified as site I and site II (Herrero and Barja, 1997). These sites generate superoxide which can be rapidly converted to H_2O_2 by superoxide dismutase. TTFA a site II specific inhibitor and Rotenone an inhibitor of Site I have been used effectively to lower mitochondria ROS production without toxic effects (Herrero and Barja, 1997). KCN, which inhibits cytochrome c oxidase (complex IV) acts distal to the source of mitochondrial ROS production (Yoshikawa and Orii, 1973). Thus KCN inhibits ATP synthesis but does not lower mitochondrial ROS formation (Forman and Boveris, 1982). Hypoxia, Rotenone, and TTFA, on the other hand, lower mitochondria-derived ROS and lower ATP production. Both of these treatments were able to stimulate HIF-1 DNA binding. HIF -1 activation by these treatments was reversed by H_2O_2 . As mitochondria are the major oxygen utilizing subcompartment of most cells, the generation of reactive oxygen species by mitochondria may serve as a cellular barometer for oxygen availability and thus could represent an ideal regulator of hypoxic signaling.

How might H_2O_2 exert inhibitory effects on the hypoxia signaling pathway? Several lines of investigation support a role for reactive oxygen metabolites and redox mechanisms in signal transduction mechanisms including modulation of protein ubiquitinylation (Jahngen-Hodge et al., 1997, Shang et al., 1997) and protein phosphorylation via regulation of a protein kinase and protein phosphatase activity (Whisler et al., 1995). Changes in the cellular glutathione and protein sulfhydryl oxidation status may also provide regulatory signals as recently demonstrated for the transcription factors NFkB, AP-1 and Sox (Fandrey et al., 1994, Flohe et al., 1997, Hidalgo et al., 1997). Indeed, the bacterial transcription factor OxyR has been shown to respond directly to redox changes associated with hypoxia (Zheng and Storz, 1998). The recently described oxygen sensitive potassium channels found in carotid body glomus cells and pulmonary vasculature smooth muscle cells may in fact also be regulated by reactive oxygen metabolites, as demonstrated by studies in which individual potassium channel subunits were expressed in *xenopus* oocytes (Duprat et al., 1995, Szabo et al., 1997). Oxygen metabolites such as H_2O_2 and hydroxyl radical may also interact with hemoprotein signal transducing enzymes such as soluble guanylate cyclase in a manner similar to CO

and nitric oxide (Schmidt, 1992). Hypoxia, by lowering reactive oxygen metabolites, might alter the binding of ROS to heme proteins and lead to a reduction in cellular cGMP or other metabolites. Such a signaling mechanism might also explain the reversal of hypoxic signaling by CO.

The second major finding of this work is not only that PKC is involved in the activation of HIF-1, but also that redox mediated signaling events take place upstream from PKC enzyme activity. Several protein kinases have been implicated in the modulation of hypoxic signaling. However, there is little consistency between these reports. The dissociation between HIF-1 DNA binding activity and hypoxia mediated transcriptional activation can be explained by the possible (and likely) involvement of multiple kinases. Since the involvement of PKC in HIF-1 transactivation was not examined in this study, any conclusions derived from this work must relate to the events which occur from the onset of hypoxic exposure to the point just beyond the nuclear translocation of the HIF-1 transcription factor. Interactions between other trans-acting factors (eg. HN-4) (Galson et al., 1995) and "auxiliary" transcriptional activators (eg. p300/CBP) have been shown to be important in transactivation of HIF-1 mediated EPO gene expression (Arany et al., 1996, Huang et

al., 1997). These interactions may require protein phosphorylation.

An argument for the role of PKC in hypoxic adaptation of cardiac tissues is supported by findings that chelerythrine blocks protection against ischemic injury (Mitchell et al., 1995, Speechly-Dick et al., 1995, Speechly-Dick et al., 1994). Also, stimulators of PKC, PDBU injury (Mitchell et al., 1995), 1-oleoyl-2-acetyl glycerol (OAG) (Speechly-Dick et al., 1994), and PMA (Ytrehus et al., 1994) confer protection. Stimulation of PKC -coupled receptors including adrenergic α_1 (Tsuchida et al., 1994), angiotensin AT_1 (Liu et al., 1995), and endothelin ET-1 receptors (Wang et al., 1996) in the rabbit heart provide protection. PKC antagonists block this induced protection. Similarly, in U87 glioma cells this work supports the involvement of PKC in HIF-1 activation --an established indicator of cellular adaptation to hypoxia. This argument will be strengthened by showing a link between PKC -coupled receptor stimulation, diacylglycerol (DAG) formation, and increased intracellular Ca^{2+} in HIF-1 protein stabilization or increased DNA binding activity. As Millhorn (1997) has shown in PC12 cells, chelation of increased intracellular Ca^{2+} blocks the hypoxic induction of tyrosine

hydroxylase mRNA production. In preliminary studies, we have also demonstrated that a combination of BAPTA/AM and Ca^{2+} free media block the hypoxic induction of HIF-1 in U87 cells(data not shown). A more definitive statement about the involvement of PKC α in HIF-1 activation will require use of an expression system in U87 subclones to examine the effects of expression of wild type and dominant negative versions of PKC α on the activation of HIF-1 by hypoxia and catalase.

In summary, the presented data supports HIF-1 activation by a reduction in ROS formation, and by PKC enzyme activity. Furthermore, since catalase activation of HIF-1 is blocked by chelerythrine chloride, the mechanism by which a reduction in cellular H_2O_2 triggers HIF-1 activation must precede PKC enzyme activity. PKC α translocation by hypoxia is also blocked by CO, placing PKC α activity downstream from the proposed heme based oxygen sensor. The proposed hypoxia sensing pathway from the sensing of molecular oxygen to the activation of HIF-1, including the above results, is expressed in Figure 23.

Figure 23: Since hypoxia results in a net decrease in ROS production, this fluctuation may serve as a cellular signal that oxygen tension has dropped. Potassium channels which are sensitive to oxygen metabolites, such as those found in pulmonary vascular smooth muscle, may acutely decrease their conductance in response to hypoxia, triggering cell depolarization. Voltage dependent calcium channels are then activated allowing an increase in $[Ca^{2+}]_i$, and stimulation of DAG formation through either phosphotidylinositol specific phospholipase C (or independently through a PLC linked receptor). PKC α is activated and phosphorylates downstream effector molecules (or HIF-1 itself) thereby promoting HIF-1 α/β translocation to the nucleus and DNA binding activity.

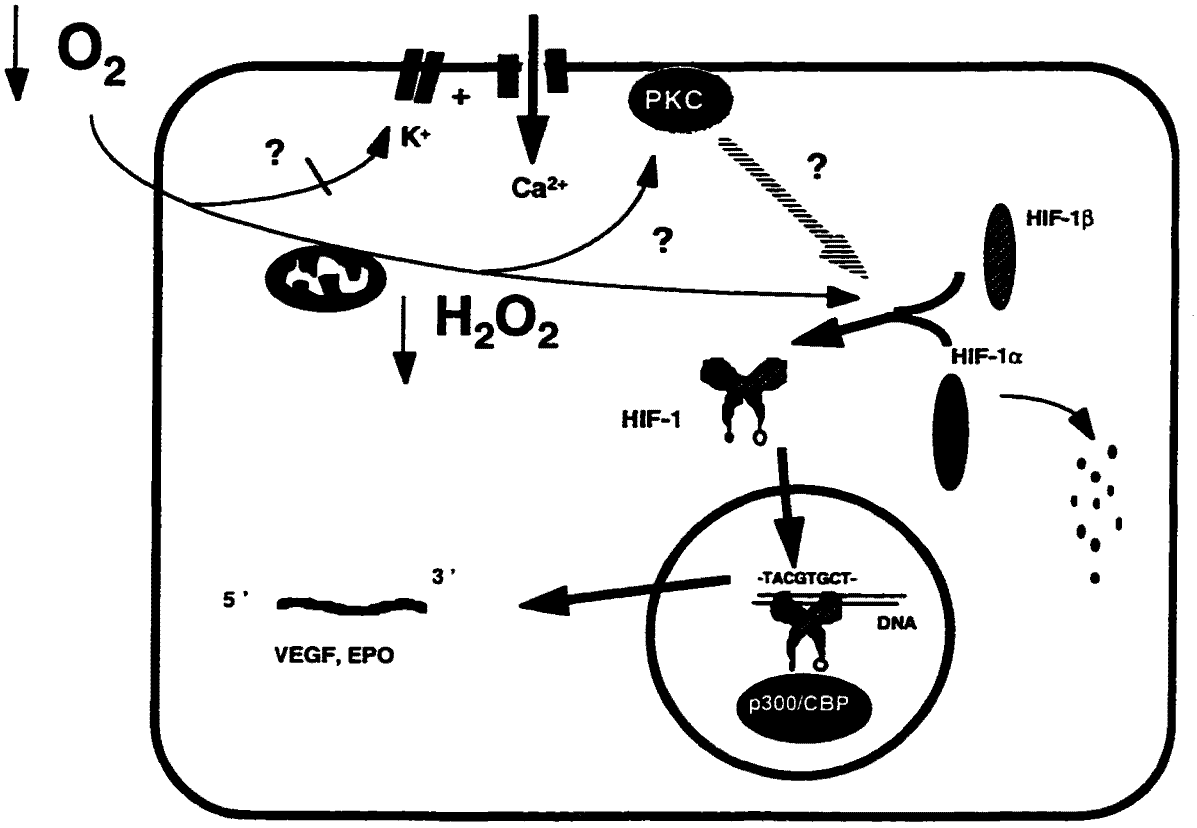

O_2
+
PKC
?
K^+
Ca^{2+}
?
?
HIF-1β
H_2O_2
HIF-1α
HIF-1
-TACGTGCT-
DNA
5'
3'
VEGF, EPO
p300/CBP

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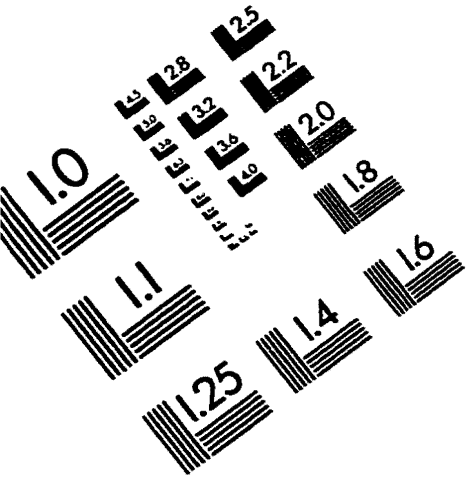
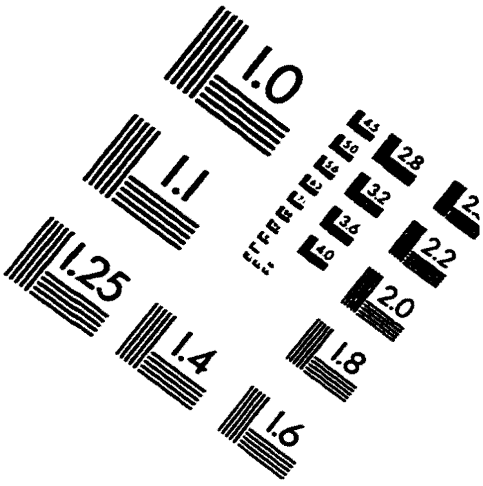
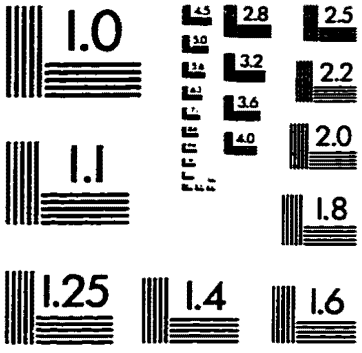
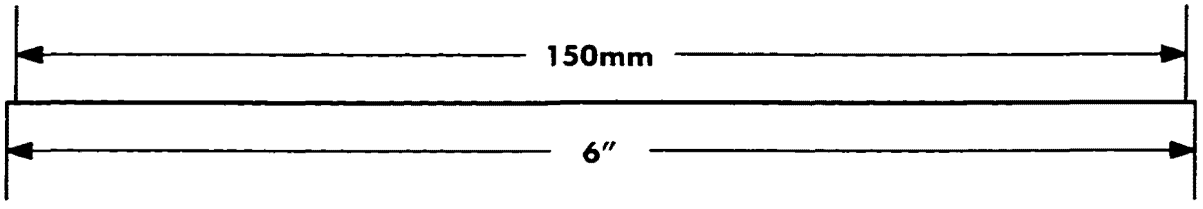
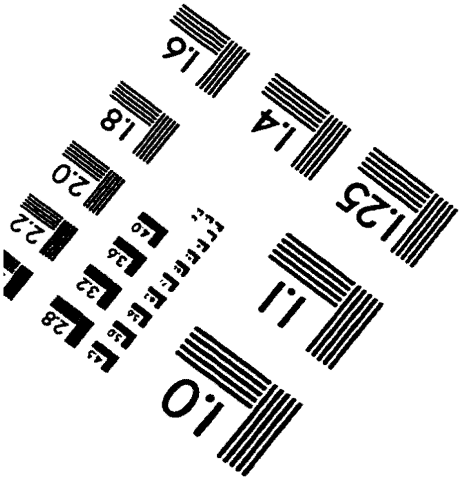
IMAGE EVALUATION
TEST TARGET (QA-3)

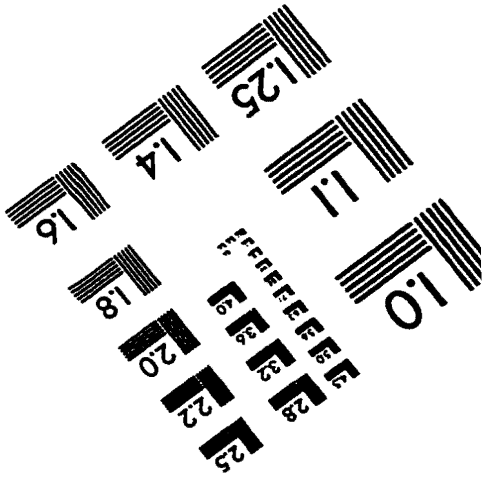












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